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THE EFFECT OF SOME STRESSORS ON THE METABOLISM OF THE

PLAICE, PLEURONECTES PLATESSA L.

THESIS PRESENTED FOR THE DEGREE OF MASTER OF PHILOSOPHY

THE OPEN UNIVERSITY

BY

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Ann White

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PUBLICATIONS

- White A. & Fletcher T.C. (1989) The effect of physical disturbance, hypoxia and stress hormones on serum components of the plaice, Pleuronectes platessa L. Comp. Biochem. Physiol. 93A, 455-461.
- White A. & Fletcher T.C. (1987) Polar and neutral lipid composition of the gonads and serum of the plaice, Pleuronectes platessa L. Fish Physiol. Biochem. 4, 37-43.
- White A. & Fletcher T.C. (1986) Serum cortisol, glucose and lipids in plaice (Pleuronectes platessa L.) exposed to starvation and aquarium stress. Comp. Biochem. Physiol. 84A, 649-653.

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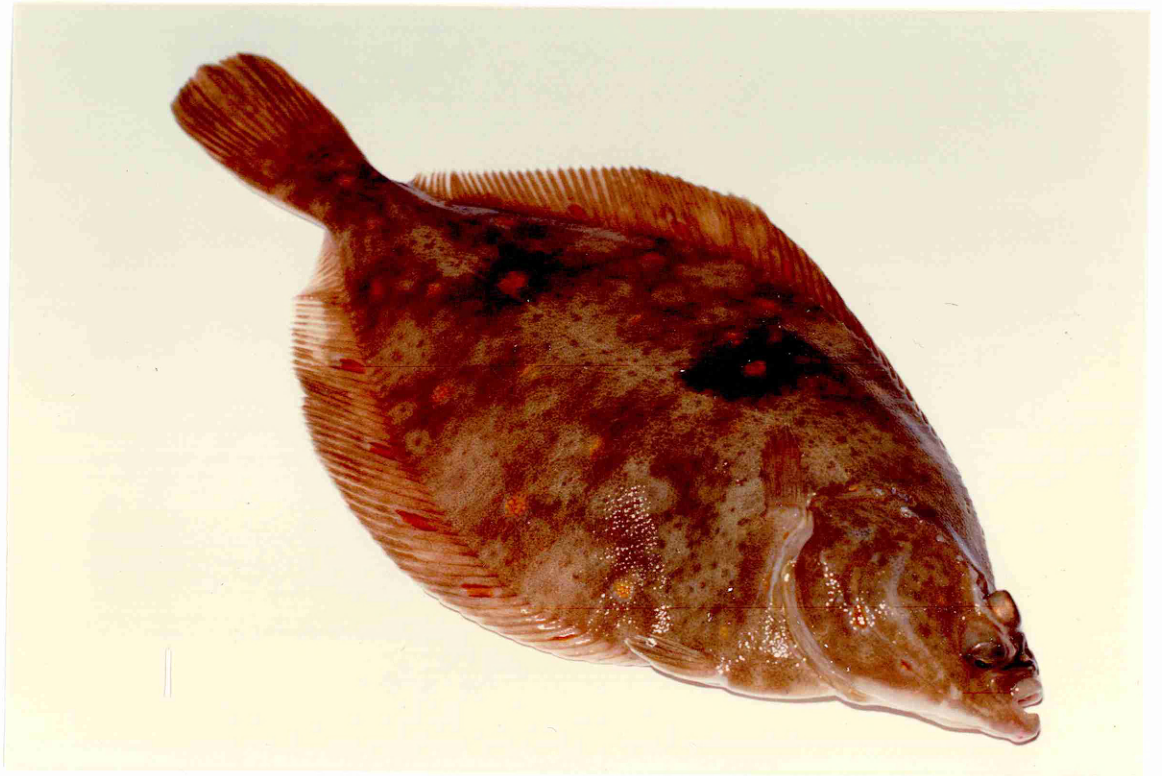
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Pleuronectes platessa L.

The influence of environmental stressors on the concentrations of various serum components was examined in the plaice (Pleuronectes platessa L.). These factors included capture, transportation and short-term starvation following arrival in the aquarium, where the effects of normal and exacerbated disturbance were also examined. The serum lipid class and concentration in male and female plaice caught at various seasons was investigated in relation to the composition of the gonads, as an example of the effects of the reproductive cycle on the nature of the circulating lipids.

Plaice were fed fresh mussel flesh for 7 months, at the end of which time, serum/plasma components were measured after 24 h without food. No significant differences were found between those values and concentrations found in wild fish 24 to 48 h post-capture (without feeding).

After capture and transportation to the aquarium, plaice were maintained for 15 days without feeding. The decrease in glucose and cortisol levels are perhaps due more to recovery from capture and transportation, rather than to lack of food.

Only cortisol levels proved sensitive to routine aquarium disturbance and in fish exposed to agitation for 1 h, but significant differences between short- and long-term physical disturbance over a 24 h period were also found for serum glucose, total cholesterol and free cholesterol. Serum non-esterified fatty acids (NEFA) concentrations, however, began to increase 3 h after the onset of disturbance, and high levels were sustained in fish from both regimes over the 24 h

period. In plaice under reduced oxygen conditions for a short period of time, increases were found in circulating levels of NEFA, cortisol, free cholesterol and lactate, but there was a decrease in the serum glucose concentration, when compared to control fish.

The intraperitoneal (i.p.) injection of cortisol had no significant effect on serum components measured after 24 h and 4 days, whereas injection of adrenalin caused an increase in serum NEFA, glucose and cortisol concentrations after 24 h and a decrease in serum NEFA after 4 days. The i.p. injection of endotoxin resulted in a decrease in serum NEFA (4 days) and glucose (24 h) levels, and an increase in serum cortisol (24 h), when compared with saline injected controls.

Total lipid, total cholesterol, free cholesterol, triglycerides, phospholipids and NEFA were measured using commercial kits, validated against standard reference methods.

I N T R O D U C T I O N

The Plaice

Natural History

The plaice, Pleuronectes platessa L was the selected experimental animal for the work described in this thesis. They live on the sandy parts of the European continental slope down to 90 m, or even 220 m in the far north, between the western Mediterranean and the White Sea, and around Iceland and the Faroes. There are a number of well-known spawning grounds where the mature fish congregate and eggs may be found in considerable numbers: off the Aberdeenshire coast is one such site. Fecundity is high with females releasing several hundred thousand eggs during the period January to March. The eggs are small (1.65-1.7 mm in diameter) and float freely, although they are well distributed between the surface and the bottom of the sea. Hatching takes place on day seventeen and the emerging fish is approximately 6.5 mm in length. Active feeding begins shortly after hatching, the food consisting of diatoms or flagellates and larval molluscs. Mortality of the newly hatched larvae is extremely high. Larval fish drift with the current and have an eye on each side of the head, but during a period of rapid metamorphosis, one eye migrates to the other side, after which the larvae settle to the bottom to assume their adult benthic existence. Normal pigmentation on adult plaice consists of a coloured ocular side, red or orange spots on a brown background, and an unpigmented underside.

Fish of the order Pleuronectiformes are unique among fish in being asymmetrical, other fish and vertebrates in general are bilaterally

symmetrical. The asymmetry is believed to have evolved from a generalised symmetrical sea bass body pattern in a fish that habitually rested on its side.

Plaice lie on the bottom generally covered by sand or mud with only their eyes protruding. The eyes can be raised or lowered and moved independently of each other. They consume a variety of molluscs, crustacea and worms with occasional fish and anemones, but predominantly bivalved molluscs. The food caught by the plaice is, of course, related to the nature and amount available on the bottom over which it presides. However, the better sense of touch in plaice as opposed to sight in dab has been used to explain why plaice eat more burrowing molluscs and dabs more of the active crustacea which must be seen rather than groped after (Wimpenny, 1953).

Absorption and Transportation of Dietary Lipids

In common with other animals, plaice require lipids as a source of metabolic energy and to maintain the structure and integrity of cellular membranes. The presence of large amounts of oil in fish, especially pelagic species, could mean that lipid rather than carbohydrate is the most likely energy reserve of fish. Dawson and Grimm (1980) reported that lipid supplies 75% of the energy for metabolism in plaice and that the main source of lipid and protein reserves is the muscle. It is known that during severe food deprivation, all animals utilise their muscle proteins in addition to lipid, as an energy source. Induced starvation of fish can result in protein stores and the free amino acid pool being utilised before

glycogen and/or lipid reserves. Prolonged starvation of fish often occurs during the annual cycle and these animals have probably evolved efficient mechanisms for dealing with this. Plaice do not feed from October, November until March, when the spawning period is past (Wimpenny, 1953).

Triglycerides, which are highly concentrated stores of metabolic energy, are important components of the natural food of many fish. Within the fish, intestinal lipases hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and non-esterified fatty acids (NEFA). An active lipase can be readily detected in fresh and lyophilised intestinal fluids from a number of marine fish (Cowey and Sargent, 1979). Patton et al. (1975) found that around 50% of the 2-monoglycerides formed during hydrolysis of triglyceride by a non-specific lipase present in intestinal fluids from several marine fish were themselves completely degraded to NEFA, whereas in the mammalian intestine, 2-monoglycerides are the major end-product of lipid digestion. Differences may exist between fish and mammals regarding the mechanism of absorption of lipid from the gut.

In mammals, the digested fats are absorbed into the lacteals and transported to the bloodstream via the lymphatic system and thoracic duct. Within the blood, lipids are transported as chylomicra to the various adipose tissues in the animal. Chylomicra particles have also been isolated from trout serum (Salmo gairdneri) (Rogie and Skinner, 1981; Sheridan et al., 1985) appearing as early as 2-4 h after feeding. However, the rapid appearance of both short- and

longer-chain free fatty acids (less than or more than 10 carbon atoms in length respectively) in the blood within 30 min, followed by the subsequent appearance of triglyceride in chylomicra suggested the possibility of a two-stage lipid absorption process for fish, only the second stage being similar to the mechanism used by mammals (Sheridan, 1988). In mammals, longer-chain free fatty acids are re-esterified on the smooth endoplasmic reticulum to 2-monoglyceride and subsequently to triglyceride, which is then incorporated into the chylomicra, whereas in fish, longer-chain free fatty acids are involved in the transportation of dietary lipids, probably bound to carrier proteins (Sheridan, 1988). Short-chain free fatty acids in both mammals and fish however, are sufficiently soluble in plasma to be transported as free solutes and leave the intestinal mucosa via the hepatic portal vein.

Another major difference exists between fish and mammals with respect to the site of storage of triglycerides. In mammals, triglycerides are mainly deposited in the adipose tissue, but in fish, fat storage is more dispersed. Some fish do possess adipose-like tissue in the viscera (Farkas, 1967), although the plaice does not, but lipids are extensively stored in the liver and muscle in plaice (Dawson and Grimm, 1980). Lipids depots in the white muscle of fish are predominantly extracellular (Cowey and Sargent, 1979), but in the liver of the plaice they are intracellular (Sargent, 1976).

Lipolysis in mammalian adipose tissue is stimulated by a number of hormones and involves activation via cyclic adenosine monophosphate.

Similar control mechanisms however, do not take place in fish adipose tissue (Farkas, 1967;1969), although a number of workers have shown that these mammalian agents do in fact stimulate liver lipase activity (Sheridan, 1988).

'Stress'

The word 'stress' features prominently in discussions of the predicament of modern man. 'Stress' however, is a situation which is not exclusive to man, as other animals, including fish, also respond to physical disruption. 'Stress' was used by Selye (1950) to describe the response made to such a force and the force itself he termed the stressor. Thus he defined stress as a reaction involving many systems of the body, including the adrenal cortex, the thymus and the digestive system and termed the series of reactions involved, the General Adaptation Syndrome. 'Stress' is perhaps better defined as "the effect of any environmental alteration or force that extends homeostatic or stabilising processes beyond their normal limit, at any level of biological organisation" (Wedemeyer and McLeay, 1981). These authors recommended that the term 'stressor' is used to describe the environmental factor itself which causes a physiological response in the fish and that terminology has been used throughout this thesis.

Stress responses are conventionally classified as primary (release of adrenocorticotrophic hormone from the adenohypophysis, and corticosteroids and catecholamines from the interrenal), secondary (blood chemistry and hematological changes, tissue and metabolic changes, and diuresis) and tertiary (impaired growth and increased

disease incidence) responses. When an animal is stressed, however, it is likely to respond in all three areas at the same time, each one being dependent on the other (Pickering, 1981). In a stressful situation in the wild, the most likely response for the fish would be to try to escape, something which is impossible for fish kept in an aquarium. This creates the added problem of unavoidable multiple stressors for experimental fish and necessity for rapid blood sampling before this action itself affects the concentrations of the blood factors being measured.

It is thought that if a fish were placed in a hostile environment, it would respond to this stressor and become adapted to the situation, so that when the stressor is removed, the fish would recover. If however, the stress situation continues too long or is too severe, the fish will eventually become exhausted, unable to cope with environmental events and probably die. Unfortunately there is no single universal response that can be measured to indicate the health of the fish. Measurement of several parameters might give more satisfactory results, but there appears to be quite a varying response according to the species involved.

'Natural Stressors'

Many factors have been reported to affect concentrations of circulating metabolites in fish, including seasonal variations in water temperature, photoperiod, gonadal development, spawning and the nutritional state of the fish (McCartney, 1966; McCartney, 1967; Leatherland et al., 1974; Delahunty et al., 1978; Farrell and Munt,

1983; Tisa et al., 1983; Fletcher, 1984). These could all be called 'natural stressors', i.e., situations which the fish would normally encounter during its lifetime. In addition to these 'natural stressors', fish maintained in an aquarium have suffered capture, handling, reduced oxygen levels and transportation, followed by more handling, perhaps increased water temperature and a completely unnatural environment.

The affect of starvation as a stressor can be illustrated by plaice, transferred from the wild to the aquarium. Once in the aquarium, plaice are unwilling to accept food and must be principally dependent on lipids, mainly triglycerides stored in the liver and muscle, being mobilised and transported to other tissues, to provide the major source of energy for vital metabolic processes. Some energy is also derived from catabolism of gut tissue (Jobling, 1980). Triglycerides, on a weight basis, contain more than twice as much energy as do carbohydrates and proteins.

Lipids and the Reproductive Cycle

Serum lipid concentrations appear to be closely related to the reproductive cycle of fish. De Vlaming et al. (1984) suggested that there was a "functional relationship" between lipid mobilisation and reproduction in staghorn sculpin (Leptocottus armatus) as they found a significant inverse correlation between visceral lipid reserves and gonadosomatic index. In the flounder (Platichthys flesus L.), serum phospholipid levels increase at the beginning of ovarian vitellogenic

activity, remaining high until after spawning (Petersen and Emmersen, 1977).

Although maximum gonadal growth in the plaice occurs at a time of non-feeding (White and Fletcher, 1985a), Dawson and Grimm (1980) found that only 22% of the stored lipid is used for gonadal development, with the muscle as the main source of lipid reserves. Teleost eggs have a varying total lipid content with values of 8-36% (Kaitaranta and Ackman, 1981) and 10-26% (Tocher and Sargent, 1984) of the dry weight of ripe ovaries. The eggs of many marine teleosts are particularly rich in phospholipids (Lasker, 1962; Kaitaranta and Ackman, 1981; Wiegand and Idler, 1982; Tocher and Sargent, 1984; Falk-Petersen et al., 1986). Tocher and Sargent (1984) reported that, in teleost eggs with an incubation period of less than 20 days, phospholipid accounted for 62-72% of the total lipid and 63-83% of this total phospholipid was present as phosphatidylcholine (PC). The incubation period for plaice eggs is 17 days (Wimpenny, 1953). Phospholipids form the major structural components of the various biological membranes that serve to compartmentalise cells and tissues and are predominantly PC, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. Phosphatidylinositol is also usually present, but in small amounts. Phospholipids are important in the metabolism of spermatozoa, as they provide necessary support to the biomembrane matrix (Lizenko et al., 1979), but there is little information in the literature on the lipid classes of marine teleost testes. In the milt of the freshwater cisco (Coregonus albula), 62-66% of the total lipid is present as phospholipid (Lizenko et al., 1973), but Belova (1982;

1983) reported that, in the spermatozoa and testes of four species of carp, phospholipid levels were 36-40% and 23-35% of the total lipid respectively. El-Sayed et al. (1984) found the triglycerides to be the major lipid class in both male and female gonads of Tilapia nilotica and the gilthead bream (Sparus auratus).

The major ovarian neutral lipid class in marine teleosts is reported to be the triglycerides (Kaitaranta and Ackman, 1981; Tocher and Sargent, 1984; Wiegand and Idler, 1985; Falk-Petersen et al., 1986), an important energy reserve for developing larvae, although cholesterol is also important as a potential source for ovarian steroidogenesis (Singh and Singh, 1979; Deb et al., 1983). For freshwater teleosts, the triglycerides and cholesterol form the major neutral lipids in the spermatozoa of the cisco (Lizenko et al., 1973) and the testes of the carp (Belova, 1983). Polar lipids are also the major lipid class in plaice serum, varying from 44-61% of the total lipid content over a 12 month period, with steryl esters the major neutral lipid (White et al., 1986). In mammals, lipids appear to have some modulating role in the immune system (Gurr, 1983) and it is possible that they could be similarly involved in fish.

Stress and Disease

Endotoxins are bacterial products implicated in bacterial pathogenesis and are classically defined as substances found inside a bacterial cell. The term is now sometimes restricted to complex substances such as proteins, polysaccharides and lipid, associated with or bound to the surface structure of a bacterial cell. The virulence of bacteria

usually relates to their capability of producing a powerful endotoxin, which interferes with normal metabolic processes, damaging host tissues and host metabolism in ways not yet clearly understood.

The life cycle of bacteria involves growth and reproduction as in other organisms. Bacteria characteristically reproduce by an asexual process, where one cell divides into two new cells. There are also instances of sexual reproduction among bacterial species. It occurs at low frequency among enteric bacteria, including Escherichia.

Escherichia coli (E. coli) is found in the intestinal tract of man and other animals and can be transmitted by hand or by water contaminated with faecal material. This can cause severe inflammation of abdominal organs and membranes. A fever may occur in addition to a rapid thready pulse, a cold sweaty skin and low blood pressure.

Plaice are frequently found around industrial and domestic sewage dumps (unpublished observation) and are therefore likely to be exposed to a high concentration of E. coli. There is a realistic chance that the bacteria will get into the gut and if exposed, the E. coli could establish well in the fish. The concentration of E. coli does reduce as the sewage is diluted but the sewage itself offers protection to the bacteria and can even support their growth (Gerba and McLeod, 1976).

Injection of bacterial endotoxins have resulted in an increase in circulating levels of cortisol in man (Kimball et al., 1968). A similar response has been reported in the plaice (White et al., 1984; White and Fletcher, 1985b) and in the rainbow trout (Wedemeyer,

1969a). Endotoxin also induces metabolic changes in plaice (White and Fletcher, 1982). Injection of endotoxin has resulted in both an increase (Kawakami et al., 1986) and a decrease (Knowles et al., 1986) in rat plasma NEFA concentrations. Knowles et al. (1986) also found that only a lethal dose of endotoxin resulted in hypoglycemia in the rat, sub-lethal doses had either no effect or caused an increase in glucose levels. Variation would appear to occur, even within the species concerned.

There is some evidence for a relationship between a stress response and susceptibility to disease in fish (Wedemeyer, 1970; Sniesko, 1974) and it is known that infections can follow a seasonal pattern in fish, for example that with trypanosomes in the plaice (Cottrell, 1977). Although seasonal variations could be linked with temperature effects on antibody production, this in turn could be influenced by the nutritional condition and hormonal balance of the fish.

'Artificial Stressors'

The stress response of teleosts resulting from various capture, handling and holding practices is well established (Hane et al., 1966; Black and Tredwell, 1967; Fagerlund, 1967; Nakano and Tomlinson, 1967; Fryer, 1975; Mazeaud et al., 1977). Tolerance to these stressors varies greatly among species (Tomasso et al., 1980; Davis and Parker, 1983) and according to the nature of the stressor (reviewed by Leatherland and Sonstegard, 1984). In many species, the stress response produces within minutes an increase in plasma catecholamines, an increase which may persist for many hours after the stressor has

been removed (Nakano and Tomlinson, 1967). This initial stress response also appears to cause an increase in adrenocorticotrophic hormone and corticosteroid levels, followed by an increase in blood lactate and glucose, and a decrease in interrenal ascorbic acid (Wedemeyer, 1981).

Physical exertion and hypoxia caused an increase in circulating corticosteroids in the coho (Oncorhynchus kisutch) and sockeye salmon (Oncorhynchus nerka), resulting in increases in blood glucose and lactate, and either an increase or decrease in NEFA depending on the species (Mazeaud et al., 1977). During hypoxia, lactic acid production is evident in many species of fish (reviewed by Van den Thillart and Van Waarde, 1985), and Jørgensen and Mustafa (1980) reported an increase in blood lactate and glucose concentrations in the flounder within 8 h of being exposed to hypoxic conditions. However, although Heath and Pritchard (1965) found an increase in blood lactate levels for the bluegill sunfish (Lepomis macrochirus) and the cut-throat trout (Salmo clarki) following severe hypoxia, these conditions produced a slight decrease in blood glucose levels in the cut-throat trout.

The response of teleosts to these stressors can also be produced by injection of adrenalin, and significant increases are evident in plaice serum cortisol (White and Fletcher, 1985b) and glucose (White and Fletcher, 1982) within 24 h. Perrier et al. (1972) found that adrenalin caused significant hypercholesterolemia in rainbow trout within 5 h of administration but had no effect on plasma NEFA levels.

Larsson (1973) however, reported significant increases in eel (Anguilla anguilla) plasma cholesterol and NEFA concentrations after the administration of 5 mg adrenalin/kg fish. The response of circulating NEFA, either to the administration of adrenalin or a stressful situation, appears to depend on the species studied (reviewed by Plisetskaya, 1980).

Purpose of This Study

Seasonal studies of circulating lipid, glucose and cortisol concentrations in relation to gonadal development and condition have already been examined in the plaice (White and Fletcher, 1984; White and Fletcher, 1985a; White et al, 1986) and this species was therefore selected for further studies. Plaice were caught off the Aberdeenshire coast by commercial fishing vessels and were available on demand. Once captured, it was very difficult to persuade the plaice to feed and starvation is another stress factor to be taken into consideration. A small number of plaice were successfully fed over a 7 month period and, although the concentrations of the serum components which were measured could not be compared to levels in wild fish, they could at least be compared to non-feeding plaice serum concentrations from fish under experimentation.

It was not possible to examine all the aspects of the stress response in plaice, so measurements of serum components were confined to cortisol (primary stress hormone) and glucose, lactate and cholesterol (secondary physiological parameters). Fish subjected to an acute stressor can suffer from a loss of ascorbic acid, cortisol,

cholesterol and other lipids in the interrenal tissue (Wedemeyer and McLeay, 1981), a loss which could be reflected in the blood. Serum lipid classes were also examined and latterly, circulating levels of ascorbic acid.

Phospholipid concentrations in plaice serum were very high, accounting for, on average, 50% of total lipid measured (White et al., 1986). The phospholipid class composition of male and female plaice serum just prior to (February/March) and after spawning (June), a period of 'natural stress' was examined and these values compared to those obtained in September when plaice appear to be in peak condition (White and Fletcher, 1985a). Phospholipid class composition was also determined in mature plaice gonads during February 1984 and March 1985 for comparison with values found in the sera. Neutral lipid classes (steryl esters, triglycerides, NEFA and cholesterol) were assayed in March 1985.

The effect of short-term starvation on serum cortisol, glucose and lipid levels was studied in the plaice. Studies on plasma hormone and metabolite levels in relation to the nutritional state of the fish have concentrated largely upon the long-term effects of food deprivation, but even the effect of handling can result in a period of non-feeding for some species (Wedemeyer, 1976; Pickering et al., 1982). The response of plaice to routine aquarium maintenance, reduced oxygen and short- and long-term physical disturbance measured over a 24 h period, was compared to the response of plaice to injected adrenalin, cortisol and endotoxin. As there is a strong possibility

that plaice could come in contact with mammalian E. coli, this bacteria was used as a source of endotoxin. It was also readily available, unlike the equivalent fish endotoxin. An inverse relationship between plasma/serum NEFA and glucose levels in teleosts has been reported in some teleosts (Farkas, 1967; Farkas, 1969; Minick and Chavin, 1973; Fletcher, 1984) and this possible relationship was examined in the plaice.

A study involving so many samples called for rapid assay methods. The recommended methods for lipid analyses are very time-consuming. Commercial kits are available for routine examination of lipids in human blood and their suitability for the estimation of plaice serum lipids was examined.

These experiments should go some way to understanding the stress response of the plaice and give some indication of the tolerance limits of this fish to changes in environmental conditions.

Fish

Plaice approximately 30 cm long, were caught by seine-net in shallow water off the Aberdeenshire coast. Groups of 10-15 fish were maintained in the aquarium in 450 litre tanks of aerated seawater at 11-13°C, usually without feeding. Blood samples were taken from the renal portal vein throughout all the experiments (Fig. 1). Portions were collected in heparinized tubes for the ascorbic acid assay and in fluoride/EDTA for the lactate assay, and the remainder was allowed to clot at 4°C. The serum and plasma were separated by centrifugation. Plaice were always killed by a blow to the head.

Plaice serum used in the lipid validation assays was obtained from fish which had been in the aquarium for 7 days. Blood samples were taken between 10.30 and 11.00 h. A commercial human serum standard (Precilip, Boehringer Mannheim, Lot No. 1-375) was assayed with each group of samples to determine the interassay coefficient of variation (CV).

Cortisol Assay

Cortisol levels in plaice serum were measured by direct radioimmunoassay (RIA) using a commercial kit (CIS UK Ltd). Commercial RIA kits for diagnosis of diseases in man are convenient, simple to use and rapid. However, human assay kits should not be used for assaying animal specimens unless validated adequately (Reimers et al., 1981). Specificity of the RIA was demonstrated in three ways: parallel inhibition curves indicated that, within the assay, the same

Figure 1 Withdrawal of blood from the renal portal vein of the
plaice, Pleuronectes platessa L.

substance in the standard solutions and samples inhibited binding of radiolabelled hormone to the antibody; the cross-reactivity of cortisol with steroids which are normally found in teleosts was not significant; the increase of cortisol in the serum after an injection of adrenocorticotrophic hormone and the decrease with dexamethsone is consistent with previously observed biological responses (White and Fletcher, 1984). The coefficients of interassay and intra-assay variations reported by White and Fletcher (1984) were within the acceptable range (Brenner et al., 1973) and essentially 100% of cortisol added to samples of serum was recovered.

This RIA method involved the use of polypropylene reaction tubes coated with specific antibody. After incubation of either 100 μ l undiluted serum or standard with radiolabelled hormone (Cortisol ^{125}I) at room temperature overnight, the unbound radiolabelled hormone was removed. Bound radioactivity was measured by an LKB Wallac 1260 Multigamma counter (White and Fletcher, 1984).

Glucose Assay

The quantitative enzymatic determination of glucose levels in plaice serum (20 μ l) was carried out using the hexokinase method, at a wavelength of 340 nm, as described in the Sigma Technical Bulletin No 15-UV. The analysis is based on the conversion of glucose to glucose-6-phosphate by adenosine triphosphate (ATP) in the presence of hexokinase, coupled with the subsequent reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine

dinucleotide phosphate, reduced form (NADPH). NADPH has a high absorbance at 340 nm, but NADP has not, therefore the reaction can be followed by measuring the increase in absorbance, against water as blank, which is directly proportional to the amount of glucose present. Glucose was also assayed using a glucose oxidase/peroxidase reagent kit (Miles Laboratories Ltd) at a wavelength of 505 nm. In this method, glucose is converted by glucose oxidase into gluconic acid and hydrogen peroxide which, in the presence of peroxidase, oxidises the chromogen (4-aminophenazone/phenol) to a red coloured compound. Serum (20 μ l) was incubated with 2.5 ml of the working solution for at least 30 min at room temperature. It is the intensity of this colour which is measured at 505 nm with the working solution as reference blank. No significant differences were found between these two methods.

Lactate Assay

Lactate levels were assayed enzymatically by incubating plaice plasma (100 μ l) with lactate dehydrogenase, nicotinamide adenine dinucleotide (NAD) and alanine aminotransferase (Test-combination Kit, Boehringer Mannheim). The absorbance of the sample was read against a sample blank at 340 nm and the concentration of lactate calculated using the formula provided.

Ascorbic Acid Assay

Ascorbic acid levels in plaice plasma were determined spectrophotometrically by the ferrozine assay at 562 nm (McGown et al., 1982). Protein was precipitated from plaice plasma (500 μ l) with 2 vol cold 10% TCA in the presence of 3.3 mM glutathione, centrifuged for 1 min in an Eppendorf high speed centrifuge and the supernatant removed. Ascorbic acid levels in the supernatants were assayed by incubating 0.4 ml of the sample supernatant in 1 ml of the ferrozine reagent (9 parts ferrozine in sodium formate plus 1 part ammonium ferric sulphate in 0.1M HCl). Samples were read at 562 nm against 0.4 ml 10% TCA in 1 ml ferrozine reagent. Glutathione was added to the TCA in order to stabilize the ascorbic acid in the plasma.

Total Lipid Assay

Plaice serum (1 ml) was extracted with chloroform:methanol (2:1 v/v) (Christie, 1982). The chloroform layer was transferred to a weighed tube and evaporated to dryness under N_2 . Tubes were kept under vacuum overnight to remove any water present and reweighed to obtain the weight of lipid extracted.

Plaice serum (50 μ l) was incubated with concentrated H_2SO_4 in a boiling water bath for 10 min. The tubes were cooled on ice, aliquots (0.1 ml) were mixed with 2.5 ml phosphovanillin reagent and allowed to stand at 20-25°C for 30 min. Lipids react with sulphuric and phosphoric acids and vanillin to form a pink-coloured complex, the absorbance of which can be read at 546 nm against a reference cuvette

containing 2.5 ml reagent plus 0.1 ml concentrated H_2SO_4 (Boehringer Mannheim test-combination kit for total lipid).

Total Cholesterol Assay

Plaice serum (0.5 ml) was saponified with alcoholic KOH and the cholesterol then extracted into petroleum ether. An aliquot of the extract was evaporated to dryness under N_2 and the cholesterol determined photometrically at 620 nm by a modified Liebermann-Burchard reagent (Abell et al., 1958; Wybenga and Inkpen, 1974).

Total cholesterol in plaice serum (20 μ l) was also measured by the CHOD-PAP (cholesterol oxidase-phenol-aminophenazone) method by reaction of the serum with cholesterol esterase and cholesterol oxidase (Boehringer Mannheim test-combination kit). Absorbance was read at 500 nm after 10 min against reagent blank.

Free Cholesterol Assay

Free and esterified cholesterol in plaice serum (1 ml) were separated by column chromatography on silicic acid (Creech and Sewell, 1962; Wybenga and Inkpen, 1974). The concentration of the free cholesterol was then determined by the modified Liebermann-Burchard reagent used for the total cholesterol assay.

Free cholesterol in plaice serum (20 μ l) was also measured by the CHOD-PAP method by reaction of the serum with cholesterol oxidase

(Boehringer Mannheim test-combination kit). Absorbance was read at 500 nm after 20 min against a reagent blank.

Triglyceride Assay

Lipids were extracted from plaice serum (1 ml) with chloroform:methanol (37:17 v/v) and the triglycerides separated from phospholipids by adsorption on silicic acid (Van Handel and Zilversmit, 1957; Rice, 1970; Wybenga and Inkpen, 1974). The triglycerides were then saponified to give glycerol, which was oxidised by periodic acid to formaldehyde. The formaldehyde concentration was determined spectrophotometrically at 570 nm by reaction with chromotropic acid.

The quantitative determination of triglycerides in plaice serum (200 μ l) was as described in the Sigma Technical Bulletin No 320-UV based on the enzymatic assay of glycerol released from triglyceride after saponification with ethanolic KOH. After saponification, magnesium sulphate solution is added to precipitate non-esterified fatty acids. The analysis is based on the formation of glycerol phosphate from glycerol with the ultimate conversion of NADH to NAD through coupled reactions. The amount of NADH oxidised, represented by a decrease in absorbance, is equivalent to the glycerol formed, which in turn, is proportional to the original triglyceride concentration. The absorbance was read at 340 nm against water.

Non-Esterified Fatty Acid Assay

Plaice serum (1 ml) was extracted with a mixture of isopropanol:heptane:0.5 M H_2SO_4 (40:10:1 v/v/v). The extract was then washed with 0.05% H_2SO_4 and the non-esterified fatty acid (NEFA) titrated with NaOH and thymolphthalein as indicator, with a stream of N_2 used for mixing (Dole, 1956; Trout et al., 1960; Wybenga and Inkpen, 1974).

NEFA in plaice serum (20 μl) were assayed by the acyl CoA synthetase-acyl CoA oxidase (ACS-ACOD) enzymatic method using the Wako NEFA C-test kit (Alpha Laboratories). NEFA in serum, when treated with ACS in the presence of ATP, magnesium cations and CoA, form thiol esters of CoA known as acyl CoA. In the second portion of the procedure, the acyl CoA is oxidised by added ACOD to produce hydrogen peroxide which, in the presence of added peroxidase, forms a purple colour with a maximum absorbance at 550 nm. Hence the amount of NEFA in the sample can be determined from the optical density measured at 550 nm against a reagent blank. Ascorbic acid in the sample could interfere in the reaction with its ability to react with hydrogen peroxide, therefore ascorbate oxidase is added to the reaction mixture at the beginning to remove all ascorbic acid from the sample.

Phospholipid Assay

Phospholipids were precipitated from plaice serum (100 μl) with TCA and oxidised to phosphate with perchloric acid and hydrogen peroxide (Zilversmit and Davis, 1950). The phosphate then formed a stable

yellow complex with molybdate and vanadate in the presence of nitric acid (Simonsen et al., 1946; Weissman and Pileggi, 1974) which could be measured spectrophotometrically.

The above method is the same as the phospholipid method supplied by Boehringer Mannheim as a test-combination kit. The absorbance was measured at 405 nm against a reagent blank.

Measurement of Polar and Neutral Lipids

Male and female gonads (1 g of wet weight or equivalent dry weight) were extracted according to Folch et al. (1957). The lipid extract was dissolved in chloroform:methanol (2:1 v/v) and stored under N₂ at -20°C until required.

An Iatroscan TH10 MarkII analyser was used to quantify the individual polar and neutral lipids (Fraser et al., 1985). Chromarod SII rods were developed in a lined chromatography tank containing either chloroform/methanol/water (70/35/3.5 v/v/v) to separate the polar lipids (Ackman, 1981) or hexane/diethyl ether/formic acid (85/15/0.4 v/v/v) to separate the neutral lipids (Kramer et al., 1980).

Polar lipids were separated by two-dimensional TLC as described by Parsons and Patton (1967), the plates sprayed with a copper acetate reagent (Fewster et al., 1969) and developed at 160°C for 40 min. This enabled the individual phospholipids to be identified.

The calibration curves used for calculating polar and neutral lipids as a percentage of the total, were supplied by A.J. Fraser (Fraser et al., 1985).

Chemicals

Unless otherwise stated, all chemicals and solvents were purchased from either Sigma (London) Chemical Company Ltd, Poole, UK or Analar Grade from BDH Chemicals Ltd, Poole, UK. All solutions were prepared in glass-distilled water.

The Boehringer Mannheim test-combination kits were obtained from BCL, Lewes, East Sussex, UK; the reagent kit for the glucose assay from Ames Division, Miles Laboratories Ltd, Stoke Poges, Slough SL2 4LY; the diagnostic kit for the glucose and triglyceride assays from Sigma (London) Chemical Company Ltd, Poole, UK; the cortisol ^{125}I radioimmunoassay kit from CIS (UK) Ltd, London, UK; and the test kit for NEFA from Alpha Laboratories, Eastleigh, Hants, UK.

Experiment 1

To Measure Serum Components of Fed Plaice in the Aquarium for 7 Months

A group of 11 plaice were fed fresh mussel flesh, ad libitum, 3 times a week. These fish were kept as undisturbed as possible over a 7 month period, from November 1985 to June 1986. Plaice were not fed for 24 h before blood samples were taken between 10.30 and 11.00 h.

Plasma lactate and ascorbic acid assays were carried out immediately, serum was stored at 4°C for use within 4 days for NEFA, total and free cholesterol and glucose assays, and the remainder at -20°C until required for the cortisol and triglyceride assays.

Experiment 2

Lipid Analysis During the Reproductive Cycle

Four groups of mature plaice (approximately 400 g gutted weight) were caught over a 13 month period, in February, June and September, and March of the following year. Plaice were allowed to recover for 7 days before blood samples were taken between 10.30 and 11.00 h. In February, gonads were removed from the fish (only 6 mature females were caught), weighed and the lipid extracted immediately. In March the following year, gonads were removed from the fish, weighed, freeze-dried and stored at -20°C until required. The gonadosomatic index for each fish was obtained by dividing the gonad weight (g) by the total body weight (g) and expressing this ratio as a percentage.

The general condition (K) was calculated as

$$K = \frac{\text{gutted weight (g)} \times 100}{\text{length (cm)}^3} \quad \text{for each fish.}$$

Plaice serum (1 ml) was extracted with 14 ml chloroform/methanol/water (4:2:1 v/v). The chloroform layer was filtered through Whatman PS 1 filter paper and dried down under N₂. The serum lipid extract was dissolved in chloroform:methanol (2:1 v/v) and stored under N₂ at -20°C until required. The remainder of the serum samples were stored

at 4°C for use within 7 days for the total lipid and phospholipid assays.

Experiment 3

The Effect of Starvation Over a 15 Day Period

The 120 fish used in this experiment were all from the same catch in May 1985. Within a few hours of capture and on arrival at the aquarium, 10 plaice were killed and bled between 15.30 and 16.00 h. The other groups of 10 fish were killed at intervals over a 15 day period. The fish were always bled between 15.30 and 16.00 h. Serum (0.6 ml) was stored at -20°C, until required for the triglyceride and cortisol assays, and the remainder at 4°C for use within 7 days for total lipid, phospholipid, total and free cholesterol, glucose and NEFA assays.

Experiment 4

The Effect of Routine Aquarium Disturbance

Plaice in this experiment were all from the same catch in August 1986 and were allowed to recover in the aquarium for 7 days before starting the experiment. Four days before the first bleed, individual plaice were marked on the white undersurface by the subcutaneous injection of 60 µl 1% alcian blue in 0.15 M NaCl (Fig. 2) and transferred to covered/uncovered tanks. Fish in the covered tanks were left undisturbed, whereas the fish in the uncovered tanks were subjected to the normal aquarium routine. After the first bleed, fish were again left in covered/uncovered tanks for 3 days until the second bleed.

Figure 2 Plaice, Pleuronectes platessa L., marked on the white
undersurface by the subcutaneous injection of 60 μ l
1% alcian blue in 0.15 M NaCl

Unfortunately during this time, some of the plaice died. Those values were not included in the mean pre-experimental values. Over the 7 day period, plaice were subjected to 4 different treatments; tanks were either covered or uncovered for the 7 days; tanks were uncovered for the first 4 days and then covered for the next 3 days, or covered for 4 days and then uncovered for the remaining 3 days.

Plaice were bled between 10.30 and 11.00 h. Serum (0.5 ml) was stored at -20°C until required for cortisol assay and the remainder at 4°C for use within 2 days for the NEFA, glucose, total cholesterol and free cholesterol assays.

Experiment 5

The Effect of Physical Disturbance

(a) Two groups of 10 plaice, caught in September 1985, were kept continuously in covered tanks for 7 days. Four days before the first bleed, individual plaice were marked as in Experiment 4. After the first bleed, fish were left for a further 3 days, when they were submitted to agitation with a net for 3 x 5 min periods, with 2 x 25 min recovery intervals. Fish were killed directly after the third agitation period and blood samples collected.

Plaice were bled between 10.30 and 11.00 h. Serum (0.5 ml) was stored at -20°C until required for cortisol assay and the remainder at 4°C for use within 2 days for the NEFA, glucose, total cholesterol and free cholesterol assays.

(b) Groups of 12-15 plaice, from the same catch in June 1986, were marked individually (as in Experiment 4). They were left undisturbed in covered tanks in the aquarium for one week. Three days before the agitation procedures, 0.5 ml blood was withdrawn from each fish. The time of this preliminary bleed coincided with the time the plaice were to be killed, so that a direct comparison could be made without the interference of any diurnal variations.

Procedure A: 6 groups of plaice were agitated with a net for 3 x 1 min periods, with 2 x 29 min recovery intervals, between 09.30 and 10.30 h. The fish were then left undisturbed until groups were killed and blood samples collected at 0, 1, 2, 4, 6 and 24 h after the third agitation period.

Procedure B: 6 groups of plaice were agitated with a net for 1 min periods, with 29 min recovery periods for a varying number of times between 09.30 and 17.00 h. Fish in the final group were submitted to a further 4 x 1 min agitation periods (between 09.00 and 10.30 h) the following day. Plaice were killed and blood samples collected at the same timed intervals as in Procedure A, with the first group killed at 10.30 h.

Serum was stored at 4°C for use within 4 days for the total and free cholesterol, NEFA and glucose assays and the remainder at -20°C until required for the cortisol assay.

Experiment 6

The Effect of Hypoxia

Two groups of 15 fish, from the same catch in August 1986, were transferred to covered tanks of aerated sea water (92-94% oxygen, measured using a Jenway oxygen meter). These plaice were left undisturbed in the aquarium for one week before starting the experiment. One group of plaice (control group) was left undisturbed, while the other group was subjected to 2 x 60 min periods in tanks containing reduced oxygen (75% oxygen), with 2 x 60 min recovery intervals. Plaice were bled immediately after the second recovery period, between 13.30 and 14.00 h. The controls were bled at the same time, to overcome any diurnal variations.

Lactate and ascorbic acid assays on plasma were carried out immediately, serum was stored at 4°C for use within 4 days for the NEFA, glucose, total cholesterol and free cholesterol assays and the remainder at -20°C until required for the cortisol assay.

Experiment 7

Simulation of the Stressors by Injection

Groups of 12-15 fish, from the same catch in August 1986, were marked individually (as in Experiment 4) and were left undisturbed in the aquarium for one week before starting the experiment. Three days before injection, 0.5 ml blood was withdrawn from each fish. All the injections and sampling were carried out between 10.30 and 11.00 h. The following agents were injected i.p. into groups of plaice (dose

0.5 ml per 300 g of fish); adrenalin bitartrate (Sigma Lot 88C-0122) dissolved in 0.15 M NaCl to give the equivalent of 200 µg adrenalin in 0.5 ml; cortisol-21-phosphate disodium salt (Sigma Lot 32F-0460) dissolved in 0.15 M NaCl to give the equivalent of 4 mg cortisol in 0.5 ml; 200 µg lipopolysaccharide B (E. coli 0111:B4 Boivin; Control No. 709536, Difco Laboratories) dissolved in 0.5 ml 0.15 M NaCl. Controls received 0.5 ml 0.15 M NaCl i.p. These plaice were bled 24 h and 4 day post-injection.

Serum was stored at 4°C for use within 4 days for the total and free cholesterol, NEFA and glucose assays and the remainder at -20°C until required for the cortisol assay.

Two further groups of plaice received either 0.5 ml of 20% glucose in 0.15 M NaCl or 0.5 ml 0.15 M NaCl. These fish were bled 6 h, 24 h and 48 h post-injection, always between 10.30 and 11.00 h.

Serum was stored at 4°C for use within 24 h for the NEFA and glucose assays.

Spectrophotometer

An SP6-550 UV/VIS (Pye-Unicam) spectrophotometer was used throughout.

Statistical Analyses

Values are expressed as arithmetic mean \pm SEM. In Experiment 7, multiple pairwise comparisons with the control were estimated by one-way analysis of variance followed by the Dunnett's multiple range

test. All other data were subjected to one-way analysis of variance and Student's t-test.

Validation of the Use of Commercial Kits

Before setting up the experiments, it was essential to establish the suitability of the chemical assays to be used for plaice serum analyses, as these had been developed for use with mammalian serum. Many reference methods for the determination of the different lipid classes are inconvenient for assaying large numbers of samples because of the initial lipid extraction procedures required. The commercial kits used throughout did not involve lipid extraction and were, therefore, quicker to use and required smaller amounts of serum.

The sulphophosphanillin spectrophotometric method for total lipids depends on the presence of unsaturated fatty acids (Wybenga and Inkpen, 1974). Should there be an increase in saturated fatty acids, this assay could yield biased data, although in teleosts, it is mainly the polyunsaturated fatty acids which are predominant at all seasons (de Vlaming et al., 1984). The sum of the lipid classes in sera from plaice caught over a 12 month period, was found to be 99% of the value for serum total lipid determined colorimetrically (White et al., 1986). This percentage was slightly higher than the 96-98% expected (Wybenga and Inkpen, 1974), but could indicate an increase in saturated fatty acids during the period March-October when the fish are feeding (Wimpenny, 1953).

Weissman and Pileggi (1974) found that serum phospholipid levels determined by the molybdivanadophosphate method were 0.2-0.3 mg/100 ml higher than by a molybdiphosphate method, but for plaice serum this increase, 0.05%, would not be significant. The initial TCA

TABLE 1 The accuracy of commercial kits (CK) as determined by comparison with reference methods (RM) for lipid analyses of plaice serum (Pleuronectes platessa L.)

	Total Lipid*		Total Cholesterol*		Free Cholesterol*		Triglyceride*		NEFA**	
	RM	CK	RM	CK	RM	CK	RM	CK	RM	CK
Plaice Serum	736 ±78	734 ±84	163 ±24	137 ±20	57 ±5	64 ±7	40 ±5	44 ±5	343 ±33	344 ±30
Human Serum Standard	653	584	106	100	31	26	134	162	-	-
Correlation Coefficient	0.990		0.991		0.878		0.986		0.965	
Probability	$\underline{P} < 0.001$		$\underline{P} < 0.001$		$\underline{P} < 0.001$		$\underline{P} < 0.001$		$\underline{P} < 0.001$	
Analysis of Variance	NS		NS		NS		NS		NS	

Plaice serum values are mean ± SEM of 10 fish

* Values are mg lipid/100 ml serum

** Values are µEqiv/L serum

NS Not Significant

precipitation in the phospholipid assay contains both protein and lipid phosphorus. In spawning plaice sampled within 1 h of capture, 20% of the TCA precipitate was present as protein phosphorus in female and 2.4% in male plasma, although this had dropped to 9% and 1% respectively after 3 weeks in the aquarium without feeding (Craik and Harvey, 1984). The influence of protein phosphorus on the female serum phospholipid levels could therefore cause an increase of approximately 10%. However, there were no significant differences between male and female phospholipid values over a 12 month period (White et al., 1986). The phospholipid mean value \pm SEM for mature plaice during February was 363 ± 12 mg/100 ml female serum ($n = 10$) and 329 ± 19 mg/100 ml male serum ($n = 10$), which are similar to values quoted by Craik and Harvey (1984). It would appear therefore that substances such as vitellogenin are not obviously influencing plaice phospholipid concentrations at the breeding season.

There was no significant difference between the reference method and the commercial kit for any of the lipid classes and only with the total cholesterol assay were the values consistently higher for the reference method. There was a close linear relationship between the results obtained by the two methods. The correlation coefficients between the methods are shown in Table 1.

The coefficients of variation (CV) for the Precilip human standard assayed by the commercial kits were all within an acceptable range (Brenner et al., 1973). The CV for total lipid was 4.88% ($n = 27$);

total cholesterol, 6.40% (n = 27); free cholesterol, 6.26% (n = 27); triglycerides, 5.09% (n = 26) and phospholipids, 6.80% (n = 25).

Experiment 1

Comparison of Fed and Non-Fed Plaice from the Aquarium

The concentrations of some serum/plasma parameters of plaice maintained in the aquarium, with feeding, for 7 months are shown in Table 2. There were no significant differences between these values obtained from plaice 24 h after their last feed in June, and those from plaice 24-48 h after capture and transportation to the aquarium from the wild in Experiment 3 caught in May the previous year.

The 24-48 h delay after capture is to allow the plaice to recover, as cortisol and glucose levels are known to be elevated at this time. Lipid levels, mainly triglycerides, will also fall during the first 48 h through the initial effect of starvation.

Experiment 2

Polar and Neutral Lipids in Plaice Sera and Gonads

The major polar lipid in both male and female plaice serum is phosphatidylcholine (PC) (Table 3). Although there were significant differences between male and female serum PC in February ($P < 0.001$), September ($P < 0.01$) and March ($P < 0.02$), there was no significant differences in either male or female PC during the year.

Phosphatidylethanolamine (PE) was only present in measurable quantities during the breeding season (February and March) and there

TABLE 2 Comparison of plasma lactate and ascorbic acid, serum glucose, cortisol and lipid concentrations in fed and non-fed plaice (Pleuronectes platessa L.)

	Fed Plaice	Days after capture without feeding*	
		1	2
NEFA (μ Equiv/L serum)	179 ± 26	182 ± 22	192 ± 29
Total Cholesterol (mg/100 ml serum)	168 ± 18	145 ± 13	137 ± 10
Free Cholesterol (mg/100 ml serum)	64 ± 8	52 ± 5	52 ± 3
Triglycerides (mg/100 ml serum)	81 ± 15	80 ± 14	58 ± 5
Glucose (mg/100 ml serum)	26.0 ± 4.7	45.5 ± 8.3	28.7 ± 1.8
Cortisol (ng/ml serum)	169 ± 32	144 ± 30	161 ± 25
Lactate (mmol/l plasma)	0.53 ± 0.01	NA	NA
Ascorbic acid (μ g/100 ml plasma)	693 ± 117	NA	NA

Values are mean \pm SEM for 11 fed fish and 15 non-fed fish

NA - not assayed

* values taken from Experiment 3

TABLE 3 Serum polar lipids (expressed as a % total lipid) in plaice, Pleuronectes platessa L.

		PE	PS+PI	PC	SM	LysoPC
1984						
	Male ^a	9.28 ±0.31	3.26 ±0.48	34.07 ±0.46	11.12 ±0.34	Trace
February						
	Female ^b	5.90 ±0.32	3.73 ±0.27	38.41 ±0.68	7.20 ±0.20	Trace
	Male ^a	ND	0.74 ±0.02	34.07 ±0.44	10.71 ±0.29	Trace
June						
	Female ^a	ND	0.75 ±0.02	35.24 ±0.56	8.37 ±0.50	Trace
	Male ^a	Trace	0.71 ±0.03	39.12 ±0.71	9.06 ±0.17	Trace
September						
	Female ^a	ND	0.62 ±0.01	36.23 ±0.63	9.37 ±0.21	ND
1985						
	Male ^a	1.12 ±0.05	0.58 ±0.03	34.28 ±0.37	8.32 ±0.26	ND
March						
	Female ^a	0.81 ±0.04	0.55 ±0.04	32.84 ±0.34	8.37 ±0.37	ND

a Values are mean ± SEM of 10 plaice

b Values are mean ± SEM of 6 plaice

ND Not detected

PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LysoPC, lysophosphatidylcholine.

were significant differences between male and female PE in both February and March ($P < 0.001$). Phosphatidylserine (PS) and phosphatidylinositol (PI) could not be separated on the Chromarods, so were calculated as PS+PI. Only in September was there any difference between male and female PS+PI ($P < 0.02$). Sphingomyelin (SM) varied little throughout the year but there were significant differences between male and female SM in February and June ($P < 0.001$). There were no measurable amounts of lysophosphatidylcholine (LysoPC) present in either male or female serum.

Plaice male and female gonads differ in that the ovaries have one major polar lipid (PC) and two major neutral lipids (triglycerides and cholesterol), and the testes have two polar (PE and PC) and one neutral (cholesterol) (Table 4). Cholesterol and diacylglycerol could not be separated on the Chromarods and because of this, the cholesterol values presented here will be slightly elevated. Although the percentage polar lipid present in the testes in March was lower than that present in February of the previous year (Table 5), the ratio PE:PC was similar; 1:0.93 (February) and 1:0.94 (March). The remaining polar and neutral gonad lipids are present in almost negligible quantities. The gonadosomatic and condition indices (Table 5) are similar to those previously reported for mature plaice during February and March (White and Fletcher 1985b).

There was no significant differences between male and female serum total lipid and phospholipid levels, except in February (Table 6). There was, however, a significant difference in the percentage polar

TABLE 4 Gonadal polar and neutral lipids (expressed as a % total lipid) in plaice, Pleuronectes platessa L.

	February 1984		March 1985	
	Male ^a	Female ^b	Male ^a	Female ^a
PE	31.76 ±0.40	9.56 ±0.40	23.64 ±1.20	5.75 ±0.31
PS+PI	0.95 ±0.06	0.75 ±0.08	0.66 ±0.04	Trace
PC	29.64 ±0.27	61.56 ±0.69	22.34 ±1.01	57.97 ±0.73
SM	6.19 ±0.11	1.29 ±0.10	6.23 ±0.22	1.39 ±0.16
LysoPC	0.76 ±0.03	0.59 ±0.04	Trace	0.68 ±0.08
SE	NA	NA	1.60 ±0.21	2.65 ±0.26
TAG	NA	NA	2.50 ±0.38	14.20 ±0.43
NEFA	NA	NA	2.46 ±0.31	1.00 ±0.18
CHOL	NA	NA	39.54 ±0.53	17.40 ±0.46

a Values are mean ± SEM of 10 plaice

b Values are mean ± SEM of 6 plaice

NA Not assayed

SE, steryl esters; TAG, triglycerides; NEFA, non-esterified fatty acids; CHOL, cholesterol.

TABLE 5 Gonadal lipid content in plaice, Pleuronectes platessa L.

	K ^c	GSI ^d	% Total Lipid (wet weight) ^e	% Dry Weight of Gonad	Total Lipid ^f	
					% Polar	% Neutral
February 1984						
Male ^a	0.90 ±0.02	1.77 ±0.21	1.64 ±0.11	11.7 ±0.4	68.5 ±1.0	31.5 ±1.0
Female ^b	0.87 ±0.02	23.8 ±2.0	1.11 ±0.12	18.7 ±0.7	73.1 ±1.7	26.9 ±1.7
March 1985						
Male ^a	0.90 ±0.03	1.96 ±0.18	1.82 ±0.21	13.3 ±0.3	52.8 ±1.9	47.2 ±1.9
Female ^a	0.91 ±0.03	24.9 ±1.9	1.80 ±0.23	26.4 ±2.4	63.4 ±1.8	36.6 ±1.8

a Values are mean ± SEM of 10 plaice

b Values are mean ± SEM of 6 plaice

c Condition Index

d Gonadosomatic index

e Determined by chloroform:methanol extraction

f Determined by the Iatroscan

TABLE 6 Serum lipid content in plaice, Pleuronectes platessa L.

		Total Lipid mg/100ml ^c	Phospholipid mg/100ml ^c	Total Lipid ^d	
				% Polar	% Neutral
1984					
February	Male ^a	935* ±54	465** ±30	55.9 ±1.7	44.1 ±1.7
	Female ^b	685* ±61	350** ±39	52.3 ±1.8	47.7 ±1.8
June	Male ^a	619 ±33	309 ±19	45.6 ±0.9	54.4 ±0.9
	Female ^a	651 ±30	303 ±12	44.3 ±1.1	55.7 ±1.1
September	Male ^a	690 ±24	364 ±19	49.3 ±1.4	50.7 ±1.4
	Female ^a	713 ±50	351 ±30	46.1 ±1.6	53.9 ±1.6
1985					
March	Male ^a	680 ±24	345 ±17	43.7 ±1.1	56.3 ±1.1
	Female ^a	627 ±44	390 ±22	41.7 ±1.2	58.3 ±1.2

a Values are mean ± SEM of 10 plaice

b Values are mean ± SEM of 6 plaice

c Assayed by test-combination kit

d Determined by the Iatroscan

* - $\underline{P} < 0.01$

** - $\underline{P} < 0.02$

lipid levels ($P < 0.005$) for both male and female plaice serum between February and March. This is reflected in the lower PE (male and female) and PC (female) levels in the sera in March (Table 3).

The ovarian polar lipid levels in both years are higher than those of the testes (Table 5), which is the reverse of the serum levels, where the male polar lipids are consistently higher than the female (Table 6).

Experiment 3

Variation in Plaice Serum Components Over 15 Days Post-Capture

The changes of some serum parameters of plaice maintained in the aquarium, without feeding, for 15 days after capture are shown in Figs 3, 4 and 5. After 24 h, the total lipid level had dropped significantly ($P < 0.001$) although it was 48 h before there was a significant decrease in the phospholipid value ($P < 0.01$) (Fig. 3).

In Fig. 4, only the triglycerides showed a significant decrease ($P < 0.001$) after 24 h. One-way analysis of variance showed that, although there was no significant variation in total cholesterol over the 15 day period, there was significant variation in the free cholesterol levels over the same period ($P < 0.005$).

NEFA and cortisol both showed a significant difference after 24 h ($P < 0.01$) and glucose after 48 h ($P < 0.001$) (Fig. 5). There was a significant inverse correlation between glucose and NEFA over 15 days ($r = -0.3687$, $n = 120$, $P < 0.001$) and between cortisol and NEFA over

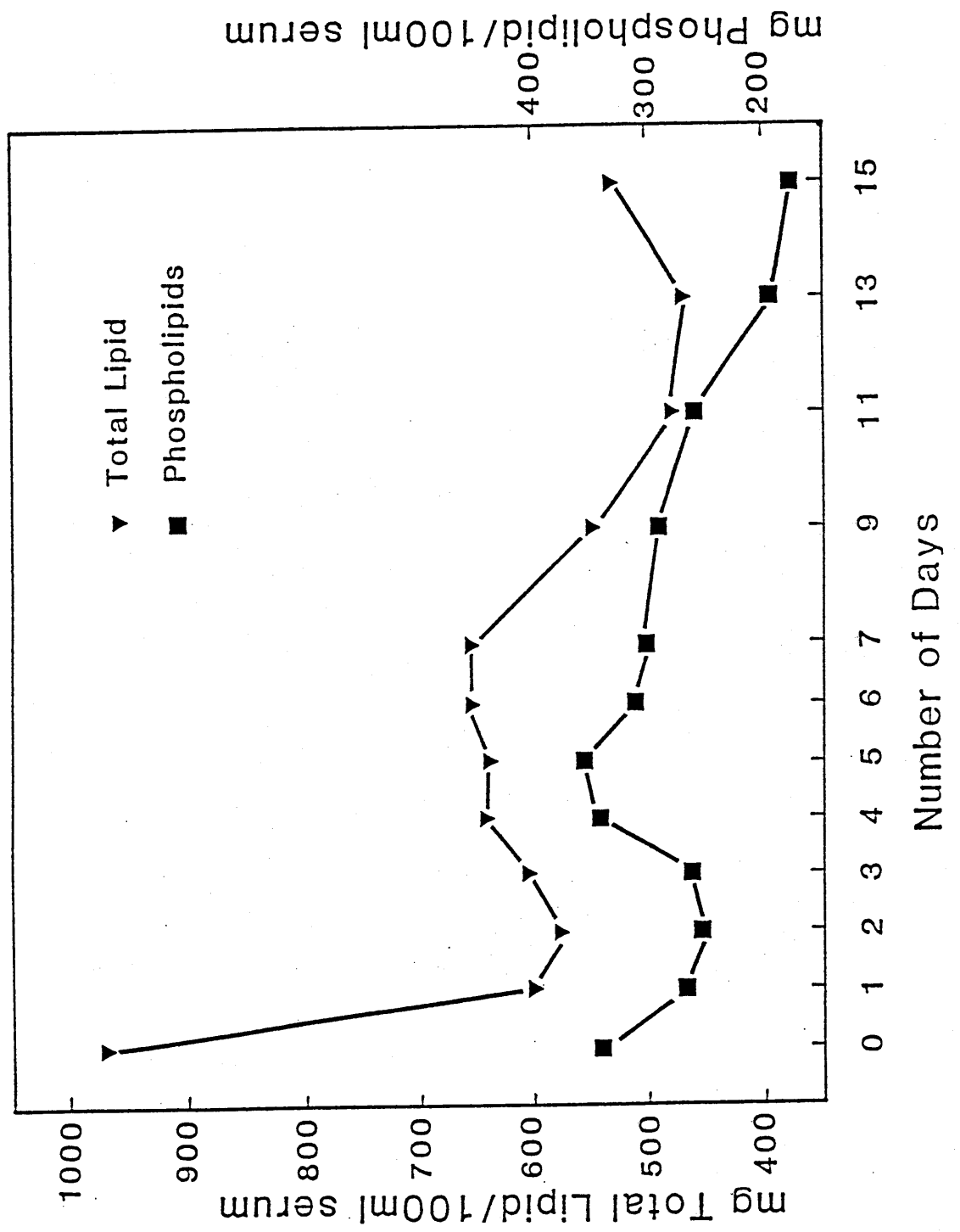


Figure 3 Serum total lipid and phospholipids from plaice
 (Pleuronectes platessa L.) kept in the aquarium and
 killed at intervals over 15 days. Values represent
 mean of groups of 10 fish, with SEM within 10%

Figure 4 Serum triglycerides, total cholesterol and free
cholesterol from plaice (Pleuronectes platessa L.)
kept in the aquarium and killed at intervals over 15
days. Values represent mean of groups of 10 fish,
with SEM within 10%

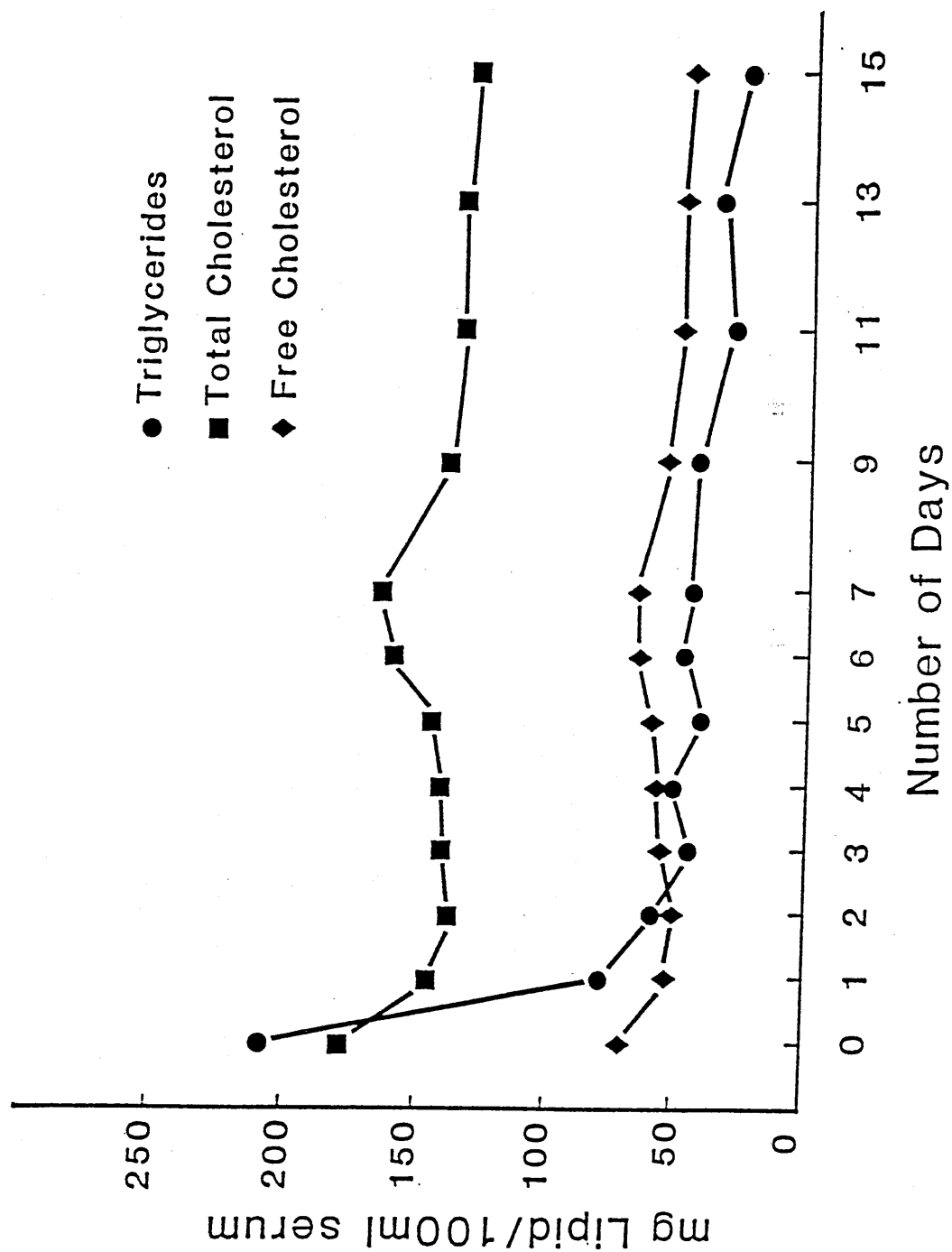
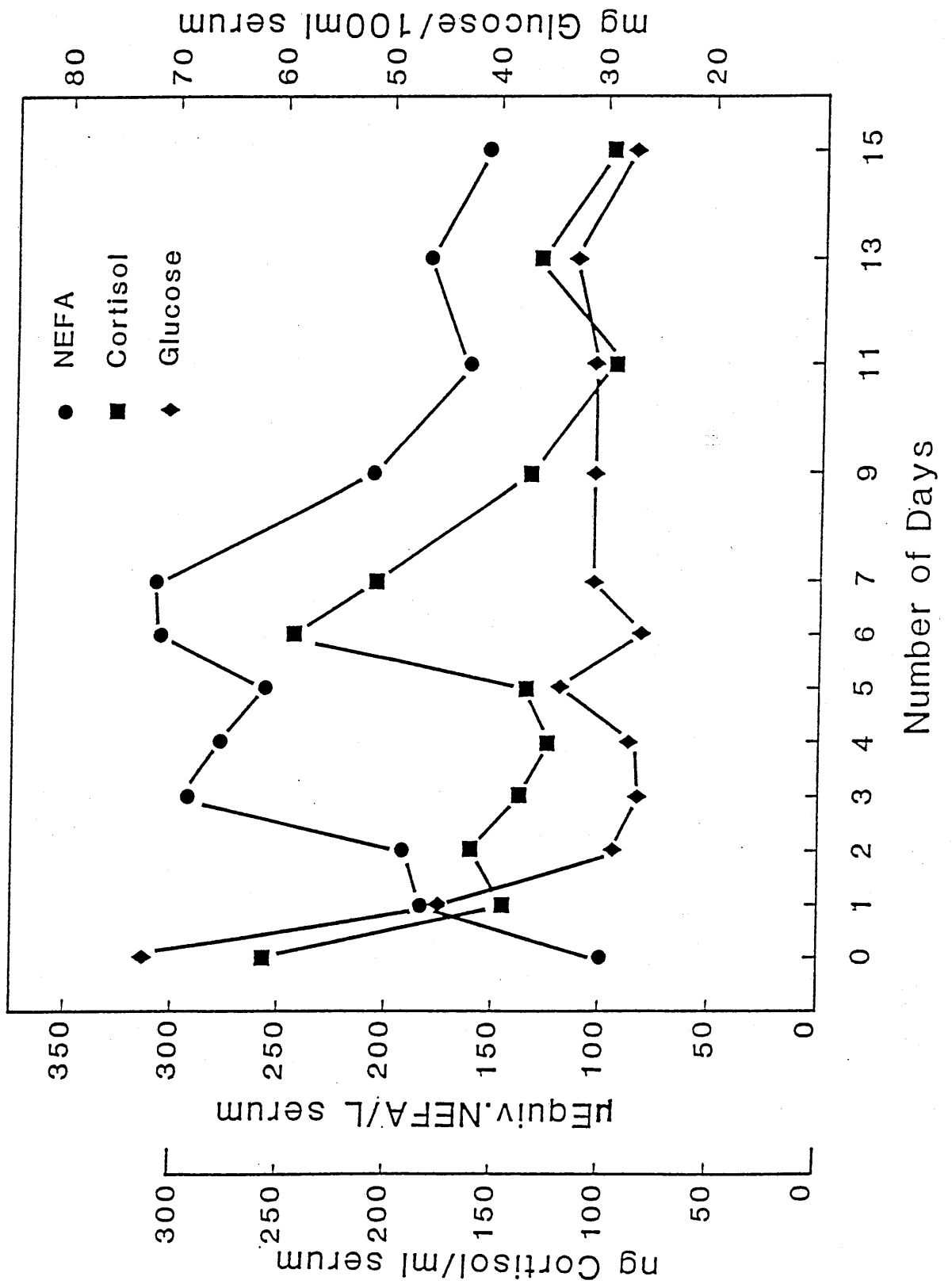


Figure 5 Serum NEFA, cortisol and glucose from plaice
(Pleuronectes platessa L.) kept in the aquarium and
killed at intervals over 15 days. Values represent
mean of groups of 10 fish, with SEM within 10%



the first 5 days ($\underline{r} = -0.3498$, $\underline{n} = 60$, $\underline{P} < 0.01$). There was also significant correlation between glucose and cortisol levels over 15 days ($\underline{r} = 0.3035$, $\underline{n} = 120$, $\underline{P} < 0.01$).

Experiment 4

The Effects of Routine Aquarium Disturbance on the Plaice

The significant differences in serum cortisol levels in the plaice caused by covering/uncovering the tanks are shown in Table 7. Covering/uncovering the tanks had no effect on the serum levels of NEFA, glucose, total cholesterol or free cholesterol.

Experiment 5

Comparison of Different Levels Of Agitation

In Experiment 5a, agitation caused an increase in plaice serum glucose ($\underline{P} < 0.05$) and a significant increase in serum cortisol levels ($\underline{P} < 0.001$) within 1 h of the start of agitation. There were no significant differences however in the serum levels of NEFA, total cholesterol or free cholesterol (Table 8).

The effect of mild and more severe stressors on plaice serum lipids, glucose and cortisol concentrations in Experiment 5b was monitored over a 24 h period. Because of the variation in the initial serum concentrations, the experimental results in Figs 6 and 7 are expressed as a percentage of the preliminary values, which are taken as 100%. The pre-experimental values for Figs 6 and 7 are given in Table 9.

TABLE 7 Serum cortisol concentrations in plaice (Pleuronectes platessa L.)
 maintained in covered and/or uncovered aquarium tanks

N*	ng Cortisol/ml plaice serum			
	Tank uncovered until 1st bleed	Tank covered until 1st bleed	Tank uncovered until 2nd bleed	Tank covered until 2nd bleed
19	94 ± 11	-	-	38 ± 9 ^b
18	161 ± 25	-	102 ± 14	-
19	-	58 ± 10	-	56 ± 10
17	-	58 ± 3	100 ± 20 ^a	-

Values are mean ± SEM

* Number of plaice per experiment

a - $P < 0.01$; b - $P < 0.001$ when compared to 1st bleed

TABLE 8 The effect of mild physical disturbance on the plaice
(Pleuronectes platessa L.) as described in Experiment 5a

	Plaice Serum Concentrations	
	Before Agitation	After Agitation
NEFA (μ Equiv/L serum)	340 \pm 23	298 \pm 22
Glucose (mg/100 ml serum)	20.3 \pm 0.9	24.0 \pm 1.5 ^a
Total Cholesterol (mg/100 ml serum)	160 \pm 10	170 \pm 12
Free Cholesterol (mg/100 ml serum)	63 \pm 3	67 \pm 5
Cortisol (ng/ml serum)	77 \pm 12	165 \pm 20 ^b

Values are mean \pm SEM of 20 plaice

a - $P < 0.05$; b - $P < 0.001$ when pre- and post-agitation values are compared

Figure 6 Serum NEFA, total cholesterol and free cholesterol concentrations, expressed as a percentage of the prebleed values of plaice (Pleuronectes platessa L.), following various times after the end of agitation procedures A and B as described under Experiment 5b in Materials and Methods. Values represent mean, with SEM within 10%. The number of fish per group are as in Table 9

1 - $\underline{P} < 0.02$; when values from Procedures A and B are compared

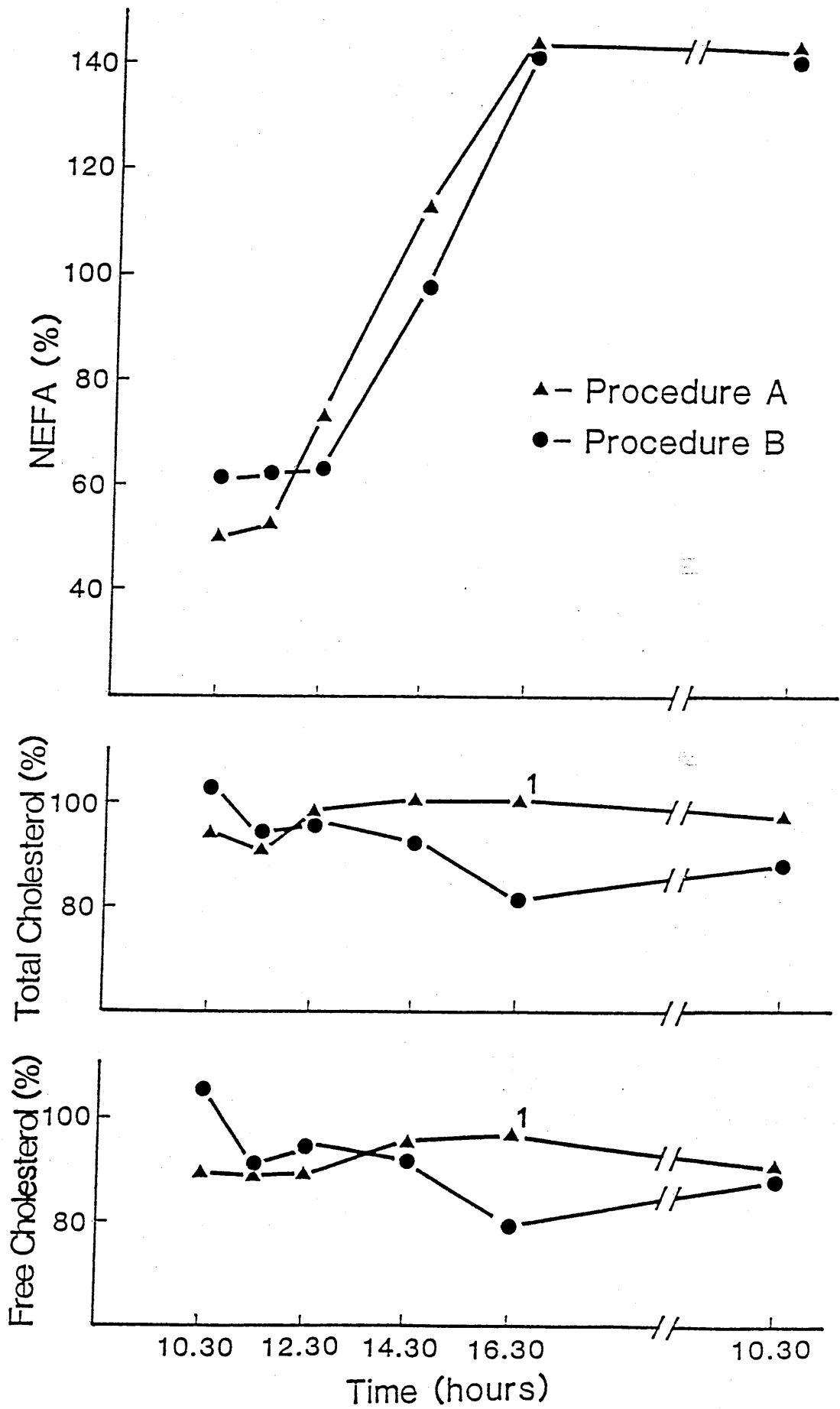


Figure 7 Serum glucose and cortisol concentrations, expressed as a percentage of the prebleed values of plaice (Pleuronectes platessa L.), following various times after the end of agitation procedures A and B as described under Experiment 5b in Materials and Methods. Values represent mean, with SEM within 10% (glucose) and 15% (cortisol). The number of fish per group are as in Table 9

2 - $\underline{P} < 0.01$; 3 - $\underline{P} < 0.001$; when values from Procedures A and B are compared

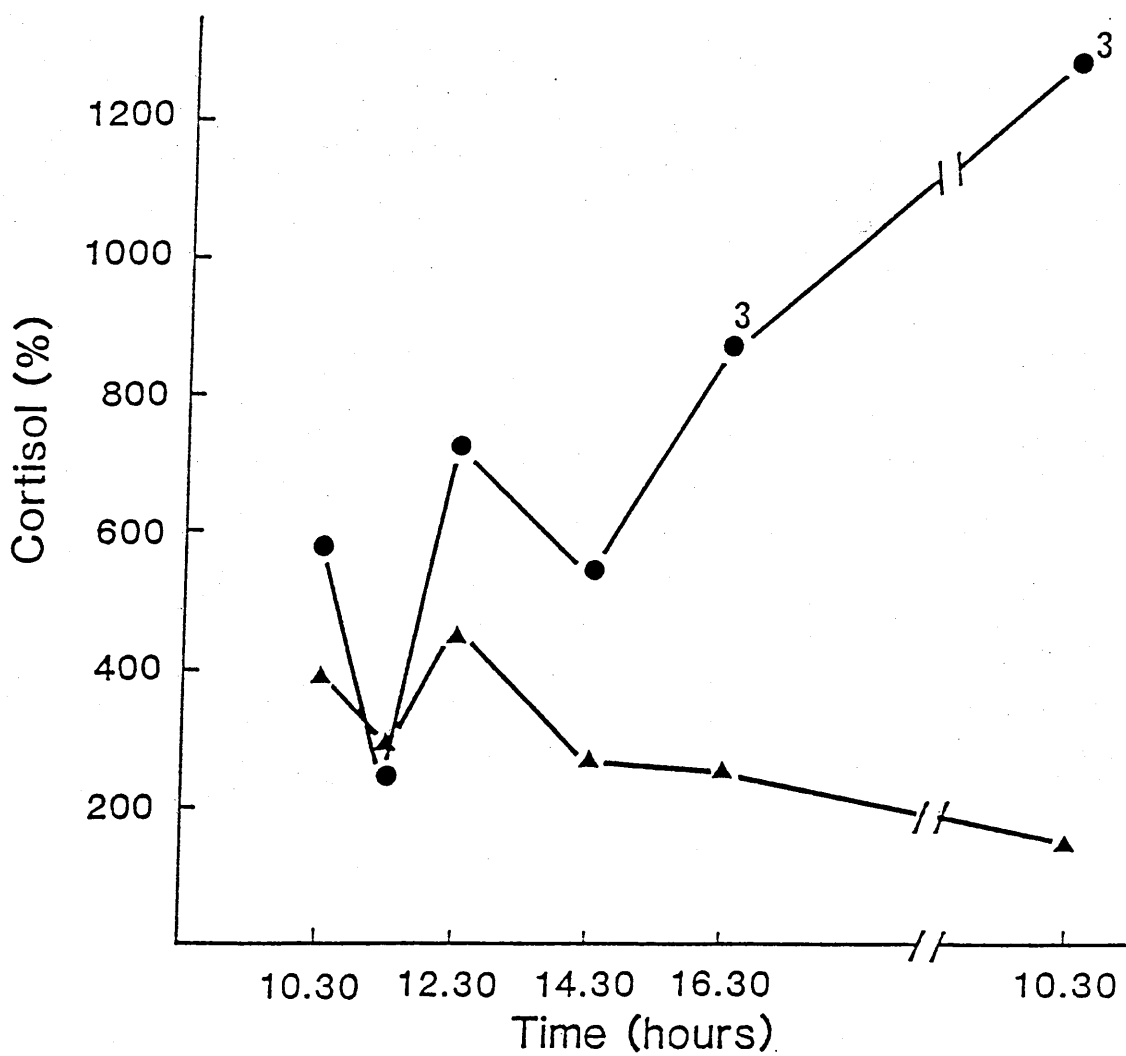
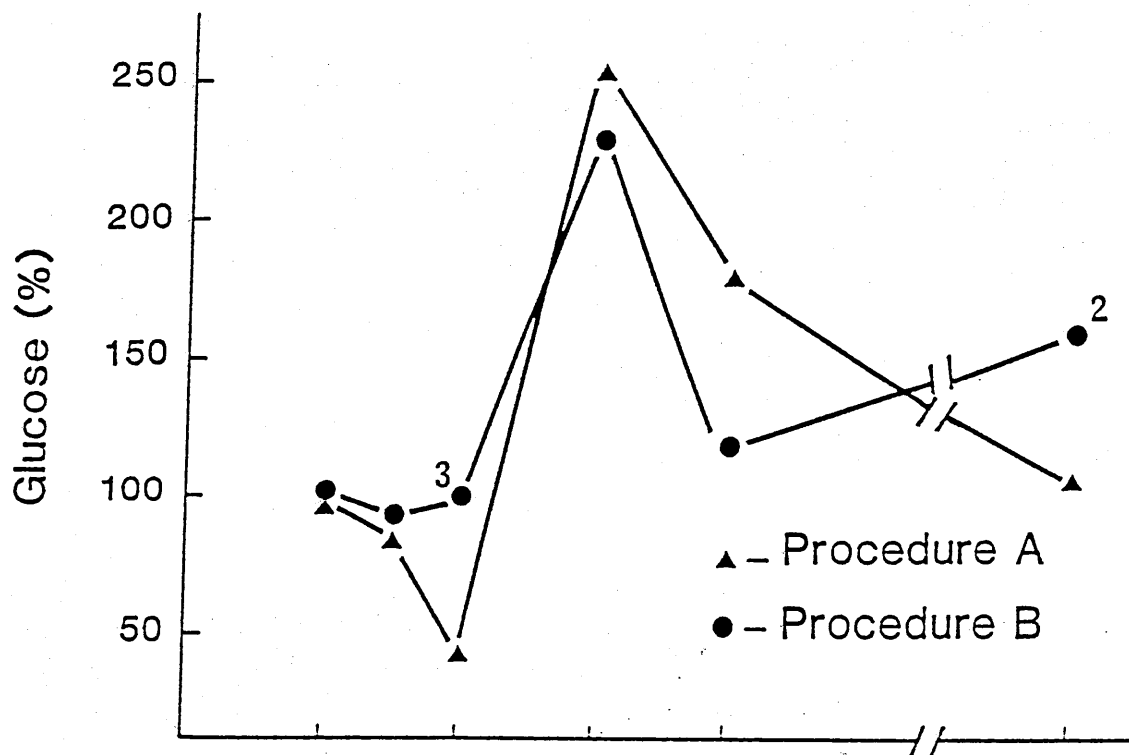


TABLE 9 Preliminary serum lipid, glucose and cortisol concentrations in the plaice (Pleuronectes platessa L.) prior to agitation by either Procedure A or B

		Time (Hours)					
		10.30	11.30	12.30	14.30	16.30	10.30
<u>N*</u>	A**	14	14	15	13	14	14
	B**	15	15	15	15	14	15
NEFA (μ Equiv/L)	A	388 ± 30	377 ^a ± 20	375 ± 27	251 ± 17	242 ± 14	281 ± 26
	B	313 ± 17	302 ± 20	333 ± 26	298 ± 18	268 ± 27	280 ± 24
Glucose (mg/100 ml)	A	18.8 ± 1.3	21.3 ± 1.4	22.7 ± 1.3	16.2 ± 1.0	20.6 ± 1.8	18.2 ^b ± 1.0
	B	17.4 ± 1.0	18.8 ± 1.5	20.8 ± 1.3	15.6 ± 1.3	19.7 ± 1.1	13.6 ± 1.2
Total Cholesterol (mg/100 ml)	A	178 ± 14	150 ± 8	161 ± 5	156 ± 7	160 ± 8	185 ± 16
	B	145 ± 10	132 ± 9	164 ± 11	151 ± 7	157 ± 9	180 ± 9
Free Cholesterol (mg/100 ml)	A	69 ^b ± 4	59 ± 2	68 ± 2	63 ± 3	63 ± 2	67 ± 5
	B	55 ± 3	52 ± 3	60 ± 4	59 ± 3	57 ± 3	63 ± 3
Cortisol (ng/ml)	A	138 ± 29	117 ^a ± 16	102 ± 23	116 ± 13	106 ± 13	108 ^b ± 14
	B	92 ± 20	194 ± 24	129 ± 27	143 ± 24	88 ± 17	49 ± 10

Values are mean \pm SEM

* Number of plaice per group

** Procedures A and B as described under Experiment 5b in the Materials and Methods

a - $\underline{P} < 0.02$; b - $\underline{P} < 0.01$; when values from Procedures A and B are compared

Plaice serum NEFA concentrations were similarly affected when fish were submitted to either Procedure A or B, with a significant decrease ($P < 0.001$), compared to pre-experimental concentrations, at 10.30 h on day 1 and a significant increase ($P < 0.01$) by 10.30 h on day 2 (Fig. 6). Serum total and free cholesterol were significantly suppressed ($P < 0.001$) by Procedure B at 16.30 h, but there was also a significant difference ($P < 0.001$) between serum free cholesterol concentrations at 10.30 h on day 1 (Fig. 6).

Serum glucose concentrations in plaice subjected to Procedure A were significantly lower ($P < 0.001$) at 12.30 h (day 1) and 10.30 h (day 2) than those obtained from Procedure B (Fig. 7). At 14.30 h, serum glucose levels in fish from Procedures A and B were both significantly higher ($P < 0.001$) than their preliminary values. Cortisol concentrations in plaice serum from Procedures A and B were not significantly different from each other until 16.30 h ($P < 0.001$), as serum cortisol in plaice submitted to Procedure A gradually decreased to near normal levels (Fig. 7). Plaice serum cortisol levels from Procedure B continued to increase 24 h after the beginning of the experiment and were significantly higher ($P < 0.001$) than cortisol levels in plaice from Procedure A at that time. All experimental serum cortisol concentrations were significantly higher ($P < 0.001$) than their pre-experimental value, except in Procedure A at 16.30 h, day 1, ($P < 0.01$) and 10.30 h on day 2 (no significant difference).

Experiment 6

The Effect of Hypoxia on the Plaice

A significant increase was observed in serum NEFA ($P < 0.01$), free cholesterol ($P < 0.02$), cortisol ($P < 0.001$) and lactate ($P < 0.001$) levels from the asphyxiated fish, but there was a significant decrease in serum glucose levels. Although the concentration of serum total cholesterol increased with the reduced oxygen treatment, the increase was not significant (Table 10).

Experiment 7

The Effect of Injected Adrenalin and Cortisol

When compared to concentrations in the saline injected fish, an i.p. injection of cortisol caused no significant changes in any of the plaice serum components measured at 24 h and 4 days post-injection (Table 11), but adrenalin, also injected i.p., caused an increase in NEFA ($P < 0.05$), glucose ($P < 0.01$) and cortisol ($P < 0.01$) concentrations in serum within 24 h post-injection, and a significant decrease ($P < 0.01$) in serum NEFA concentrations 4 days post-injection (Table 11).

The Effect of Injected Endotoxin

The i.p. injection of endotoxin, again when compared to the control values, also produced a significant decrease in serum NEFA concentration 4 days post-injection ($P < 0.05$), with a decrease in serum glucose levels ($P < 0.01$) and an increase in cortisol levels

TABLE 10 The effect of reduced oxygen treatment on serum/plasma components of the plaice (Pleuronectes platessa L.)

	Control	Experimental
NEFA (μ Equiv/L serum)	363 \pm 21	485 \pm 28 ^b
Total Cholesterol (mg/100 ml serum)	237 \pm 19	319 \pm 30
Free Cholesterol (mg/100 ml serum)	80 \pm 5	105 \pm 8 ^a
Triglycerides (mg/100 ml serum)	51 \pm 3	66 \pm 6
Glucose (mg/100 ml serum)	17.8 \pm 2.2	8.9 \pm 2.7 ^a
Cortisol (ng/ml serum)	137 \pm 24	417 \pm 72 ^c
Lactate (mmol/l plasma)	0.6 \pm 0.1	16.7 \pm 2.0 ^c
Ascorbic Acid (μ g/100 ml plasma)	519 \pm 50	642 \pm 42

Values are mean \pm SEM of 15 plaice

a - $\underline{P} < 0.02$; b - $\underline{P} < 0.01$; c - $\underline{P} < 0.001$; when control and experimental values are compared

TABLE 11 The effect of injected saline, cortisol, adrenalin and endotoxin on serum components of the plaice (Pleuronectes platessa L.)

Treatment	mg Cholesterol/ 100 ml serum		mg Glucose/ 100 ml serum	mg Cortisol/ ml serum	
	Total	Free		Total	Free
<u>3 days prior to injection</u>					
Saline	350 ± 34	15.8 ± 1.5	263 ± 19	120 ± 11	103 ± 18
Cortisol	375 ± 23	15.2 ± 1.1	249 ± 18	114 ± 11	117 ± 18
Adrenalin	372 ± 40	17.0 ± 1.0	254 ± 31	109 ± 17	147 ± 31
Endotoxin	448 ± 35	15.4 ± 0.7	277 ± 16	113 ± 8	150 ± 20
<u>24 h post-injection</u>					
Saline	410 ± 25	16.5 ± 0.9	224 ± 13	95 ± 7	128 ± 27
Cortisol	447 ± 38	18.3 ± 0.7	207 ± 16	88 ± 18	1748 ± 11 ^b
Adrenalin	524 ± 47 ^a	163.4 ± 38.6 ^b	277 ± 43	119 ± 23	574 ± 51 ^b
Endotoxin	338 ± 28	7.3 ± 0.6	222 ± 12	87 ± 6	269 ± 36 ^a
<u>4 days post injection</u>					
Saline	433 ± 35	16.0 ± 1.8	211 ± 12	80 ± 5	175 ± 21
Cortisol	388 ± 40 ^b	15.8 ± 1.5	209 ± 18	79 ± 8	765 ± 77
Adrenalin	279 ± 29 ^b	14.0 ± 2.5	178 ± 23	70 ± 11	183 ± 27
Endotoxin	299 ± 24 ^a	11.3 ± 0.9	206 ± 13	78 ± 7	196 ± 34

Values are mean \pm SEM of 15 plaice
a - $P < 0.05$; b - $P < 0.01$ when compared with saline injected controls

($P < 0.05$) within 24 h of injection (Table 11).

The Effect of Injected Glucose

Within 6 h of an i.p. glucose injection, circulating glucose levels had significantly increased ($P < 0.001$), remaining high ($P < 0.01$) at 24 h, before returning to normal levels by 48 h (Table 12). There was also a significant increase in glucose levels ($P < 0.01$) in serum from the saline injected plaice. The injected glucose however, had no obvious effect on plaice serum NEFA concentrations (Table 12).

TABLE 12 The effect of injected saline and glucose on serum NEFA and glucose levels in the plaice (Pleuronectes platessa L.)

	Saline Injected		Glucose Injected	
	μ Equiv NEFA/ L serum	mg Glucose/ 100 ml serum	μ Equiv NEFA/ L serum	mg Glucose/ 100 ml serum
3 days before injection	353 \pm 13	11.9 \pm 1.0	329 \pm 21	10.5 \pm 0.7
6 h post-injection	353 \pm 22	19.3 \pm 1.7	304 \pm 19	55.7 \pm 3.7
24 h post-injection	380 \pm 21	11.9 \pm 1.3	313 \pm 25	28.3 \pm 5.2
48 h post-injection	347 \pm 26	9.8 \pm 0.9	306 \pm 23	10.7 \pm 1.3

Values are mean \pm SEM of 14 plaice

DISCUSSION

Normal Serum Values

As wild plaice are difficult to feed once transported to the aquarium, they are, of necessity, maintained without feeding, so that the levels of various serum components measured could not be directly compared to those from feeding fish. Eleven plaice accepted fresh mussel flesh and were thus maintained in the aquarium for 7 months, during which time they were subjected to the routine aquarium disturbance, hence the elevated cortisol levels as shown in Experiment 4. The remaining serum values corresponded to those of recently caught plaice, when comparisons were made between plaice in June (aquarium) and May (24 and 48 h after capture, Experiment 3). This should then give some idea of the levels expected in plaice before artificial stressors were applied, ie as normal as can be expected with fish being kept in the aquarium.

With the exception of plaice cortisol levels, which were slightly higher than reported values for other teleosts, all other serum/plasma components concentrations examined in this study were similar to published values for teleosts.

Plaice serum NEFA values used during this work ranged from 179-448 μ Equiv/L. A wide range of serum values for teleosts has also been reported in the literature. Larsson and Fänge (1977) reported a mean of 567 μ mole/L for newly caught plaice and Fletcher (1984) found values from 390-935 μ Equiv/L over one year for the dab (Limanda limanda). In a review of marine teleost NEFA values, Plisetskaya (1980) found values ranging from 180-1540 μ Equiv/L.

The variation in plaice serum cortisol levels in these experiments is also wide ranging, from 49-169 ng/ml. Strange (1980) reported a serum cortisol level for channel catfish (Ictalurus punctatus) of 25 ng/ml, Pickering (1982) 25 ng/ml for brown trout (Salmo trutta), and for rainbow trout, Zelnik and Goldspink (1981) 70 ng/ml, Leatherland (1985) 46-113 ng/ml and Woodward and Strange (1987) 2-10 ng/ml.

Plaice serum glucose values were less scattered with values from 10.5-26.0 mg/100ml. Wardle (1972) reported 15-25 mg/100 ml and Larsson et al. (1976) 34.5 mg/100 ml for glucose levels, also in the plaice. Jørgensen and Mustafa (1980) found glucose levels of 24 mg/100 ml in the flounder and Fletcher (1984) 20-25 mg/100 ml in the dab.

Total cholesterol levels in plaice serum varied from 145-277 mg/100 ml. A review by Hille (1982) gave values for rainbow trout ranging from 150-545 mg/100 ml. Coho salmon were reported to have levels of 260-620 mg cholesterol/100 ml depending on site and sex (Leatherland and Sonstegard, 1984). Larsson and Fänge (1977) found the mean cholesterol value for newly caught plaice to be 658 mg/100 ml, with 68% present as cholesterol ester. By difference, cholesterol esters were present in this study in the range 54-62%.

On arrival at the aquarium after capture, the mean triglyceride value for plaice was just over 200 mg/100 ml. Fish maintained in the aquarium however, had much lower values ranging from 51-81 mg/100 ml. Triglyceride values for rainbow trout were reported in the range

107-603 mg/100 ml (reviewed by Hille, 1982), and Leatherland and Sonstegard (1984) found values of 68-435 mg/100 ml in the coho salmon.

Mean plasma lactate levels in plaice were 0.53 and 0.6 mmol/l.

Wardle (1972) reported values in the range 0.5-2.0 mmol/l, and Larsson et al. (1976) 2.5 mmol/l, also for plaice plasma. Plasma lactate values for rainbow trout were in the range 0.15-1.92 mmol/l (reviewed by Hille, 1982) and for brown trout, 1.11 mmol/l (Pickering, 1982).

Lipids and the Reproductive Cycle

Lipids such as PC and PE, which are soluble in both water and fats, serve a vital role in the cell by binding protein to lipids. PC is an important structural component of the cell membrane because its hydrophilic groups maintain continuity between water inside and outside the cell and its hydrophobic groups allow lipid material to enter the cell. PS and PI are not present in large amounts and are minor components of cells compared to PC and PE. Cholesterol, with phospholipids, also plays an important role in the membrane structure of cells and is the precursor for many other biologically active sterols, ie the steroids.

Triglycerides and fatty acids are formed during the digestive processes in animals and, if an animal ingests more than it can utilise in energy requirement functions, excess fatty acids combine with glycerol to form neutral lipids, which are then stored in the animal. These stored neutral lipids can then be used at a time of

non-feeding, just prior to and during spawning and could also serve as a food reserve for the developing ovaries.

Earlier work had indicated seasonal changes in plaice lipids (White et al., 1986) and a more detailed examination was made of changes in lipid composition and the relationship with the reproductive cycle.

Serum Lipids

Although total serum cholesterol levels in the plaice are only slightly higher than in man, serum phospholipid is present in much higher concentrations. In plaice serum, PC represented approximately 70% of the total polar lipid and this percentage is similar to that found by Bolis and Fänge (1979), who examined the phospholipid distribution in fish erythrocyte membranes and reported that PC accounted for approximately 60% of the polar lipids in all the teleosts studied. Tocher and Sargent (1986) however, reported that in plaice neutrophils, while 48% of the total lipid is present as polar lipid, PE is present at concentrations similar to PC, and the triglycerides are the major neutral lipid class.

For plaice serum in this study, the major neutral lipid class for male and female is the steryl esters; in February and March, steryl esters represented 25 and 28% respectively of the total lipid present in male serum and 31 and 35% respectively in female serum (unpublished observation). With the variation in the literature, the neutral lipid distribution appears to be dependent on the sex and species examined. For the White Sea flounder (Platichthys flesus bogdanovi), cholesterol

and steryl esters are the major neutral lipids in both male and female serum (Lapin, 1973). Triglycerides and steryl esters are the major neutral lipids in the male serum of the Baltic cod (Gadus morhua callarias), but in the female serum, the major neutral lipid is the triglycerides (Shatunovskiy, 1971).

Ovarian Lipids

The occurrence of PC as the major polar lipid in plaice ovaries, is in agreement with published reports for the ovaries from other marine teleosts (Lizenko et al., 1979; Tocher and Sargent, 1984; Cowey et al., 1985; Falk-Petersen et al., 1986). Cowey et al. (1985) reported that, during the 138 days after fertilisation, the level of PC in salmon (Salmo salar) larvae gradually decreased, indicating its use as an energy source. Tocher et al. (1985) speculated that PC was also an energy reserve for Atlantic herring (Clupea harengus) eggs and larvae, as did Falk-Petersen et al. (1986) for halibut (Hippoglossus hippoglossus) and this could be the reason for high levels of PC in plaice ovaries.

The major ovarian neutral lipid again appears to depend on the species examined, but for plaice, cholesterol and the triglycerides were the predominant classes, with a cholesterol:triglyceride ratio of approximately 1:1. This ratio is similar to that reported for the haddock (Melanogrammus aeglefinus) and saithe (Pollachius virens) (Tocher and Sargent, 1984), the flounder (Pseudopleuronectes americanus) (Wiegand and Idler, 1982) and the Atlantic halibut (Falk-Petersen et al., 1986).

Lipids in the Testes

The major polar lipids in plaice testes were PC and PE, which were present in a ratio of almost 1:1. Similar results were found for the sperm of rainbow trout (Minassian and Turner, 1966) and the cisco (Lizenko et al., 1979) where the PE:PC ratio was 1:1.5 and PE and PC were the major polar lipids.

The major neutral lipid class in the testes also seems to depend on the species studied and in the plaice, it is the cholesterol class which predominates. Cholesterol is also reported to be the major neutral lipid class for trout spermatozoa (Minassian and Turner, 1966) and the lane snapper testes (Lutjanus synagris) (Klaro and Lapin, 1971). Lizenko et al. (1979) suggested that the total mass of plasma membranes, where cholesterol, together with SM, is mainly concentrated, is higher in the testes than in the ovaries. This could explain why levels of cholesterol and SM are higher in plaice testes than in the ovaries.

Variation of Lipids in Plaice

Although PC was found to be the major polar lipid in plaice serum and ovaries, the high level in the ovaries is not reproduced in the female serum. The high level of PE in plaice testes is also not reflected in male serum, where the percentage of PC is higher than that in the testes. These differences are difficult to explain, but the phospholipid composition of biological membranes has been found to vary, not only with species, but with the tissue of origin (McMurray,

1973). This could perhaps contribute to the variation in PC and PE values reported here. Another explanation may be, that in the ovaries it is PC which is the lipid used in protein binding, but in the testes it is PE. As the lipoprotein would then accumulate during maturation of the gonads, the high levels would not necessarily be reflected in the serum.

When compared with the previous year, the March decrease in the proportion of polar lipids of the total lipid in both gonads and serum, is also difficult to explain. Lizenko et al. (1979) found that the lipid class composition of the eggs of the cisco, particularly the triglycerides and steryl esters, varied from year to year, depending on the lipid composition and availability of the food supply. The study of Di Costanzo et al. (1983) reported that dietary fatty acids affected the polar lipid class composition of trout intestinal brush border membrane. It is therefore possible that the qualitative composition of the food supply (i.e. the fatty acids available for phospholipid synthesis), is one of the environmental factors dictating the lipid composition of the plaice. Also, lipid levels in the muscle and liver stores of fish can change with the nutritional state of the animal, giving marked seasonal differences (Sargent, 1976) and this would presumably cause the serum lipid levels to vary accordingly.

Capture and Transportation

The effects of capture and transportation on some serum components of the plaice and their variation over a period of 15 days in the aquarium were studied. Care was taken when sampling the fish, as both

glucose and cortisol levels can easily be affected by handling (Leatherland and Sonstegard, 1984) and this could complicate the interpretation of sequential serum changes thought to be due to the initial Day 0 treatment. The effects of these stressors on teleosts are well documented (Hane et al., 1966; Black and Tredwell, 1967; Fagerlund, 1967; Nakano and Tomlinson, 1967; Fryer, 1975; Mazeaud et al., 1977), although tolerance to the stressors varies greatly among species (Tomasso et al., 1980; Davis and Parker, 1983).

In the plaice, only circulating glucose and cortisol concentrations were elevated in response to capture and transfer to the aquarium, the fish requiring 24-48 h to acclimatise to their new environment. Wingfield and Grimm (1977) reported that plaice plasma cortisol fell to a minimum within 2 days post-capture and Wardle (1972) that, after capture, plaice required 2-3 days to acclimate to their new environmental conditions, after which there was little change in the blood glucose levels. The high value for plaice serum glucose on Day 0 must therefore reflect the stress response to capture and transportation (Carmichael et al., 1983) and as the values obtained from the fed plaice were very similar to those 24-48 h post-capture, this would confirm that speculation. This hyperglycemic response is probably due to an increase in both gluconeogenesis and glycogenolysis in the body tissues.

With the exception of plaice serum NEFA, there was a sharp decline in the initial levels of circulating lipids in the 24 h following arrival in the aquarium, with only slight variation after 48 h. This initial

decrease could be due more to lack of feeding than to the stress response to capture and transportation. Contrary to what was expected, serum NEFA levels began to rise after arrival in the aquarium. One possible explanation is the mobilisation of NEFA through increased lipase activity to compensate for the lack of feeding. The values 24 and 48 h post-capture however, are very similar to those of the fed fish.

Starvation over a 15 Day Period

There was very little variation in the plaice serum glucose levels after 48 h in this study and it would appear that lack of food over a short period of time does not appear to have an effect on fish serum glucose. This is in agreement with other reports in the literature (Chavin and Young, 1970; Zammit and Newsholme, 1979; Fletcher, 1984; Lewis and Epple, 1984). By contrast to omnivorous animals, fish do not mobilise liver glycogen rapidly when they are starved. Nagai and Ikeda (1971) found that blood glucose and liver glycogen levels in carp which had been starved 22 days, were not significantly different from fed fish. It has also been suggested that the demands of tissues such as brain and nervous tissue, which catabolise glucose as a primary fuel are met by gluconeogenesis rather than by glycogenolysis, blood glucose levels being maintained by the former process (Cowey and Sargent, 1979).

Plaice serum cortisol levels initially fell post-capture, then rose to a peak at 6 days. This is similar to the variation in plaice plasma cortisol levels found by Wingfield and Grimm (1977), with a peak at

10 days. There was no reported change for serum cortisol levels in Fundulus heteroclitus after fasting for 35 days (Leach and Taylor, 1982) or in plasma cortisol levels in starved rainbow trout sampled at intervals up to 65 days (Milne et al., 1979).

After 24 h in the aquarium, there was little change in the total lipid, phospholipid and triglyceride levels in plaice serum over the 15 day period. There was little change in eel (Anguilla rostrata) serum cholesterol after 6 months starvation (Lewis and Epple, 1984) and no difference in the char (Salmo alpinus) plasma total and free cholesterol after starving for three weeks (Dannevig and Norum, 1983). For bass, Dicetrarcus labrax (Zammit and Newsholme, 1979) and char (Dannevig and Norum, 1983) there was a slight decrease in plasma triglycerides over the period studied. Craik and Harvey (1984) found a slight decrease in plaice plasma lipid phosphorus after 3 weeks in the aquarium without feeding. Plaice can survive several months without feeding over the winter months, so they probably have a very efficient metabolism to control the lipid release over a period of non-feeding.

Farkas (1967) reported that plasma NEFA concentrations are normally dependent on the nutritional state of the fish and increase with starvation. Plaice serum NEFA increased significantly over the first 3 days without food confirming the importance of serum NEFA as an energy source for teleosts (Zammit and Newsholme, 1979). This is probably due to an increase in hepatic triglyceride lipase activity. Plaice serum NEFA levels reached a maximum at 7 days and required

9-11 days to stabilise. The decrease between 7 and 11 days post-capture (57%) could explain the initial decrease in serum NEFA concentrations in plaice subjected to both Procedures A and B in Experiment 5b. Similar results were found for plasma NEFA concentrations in the sand dab which increased significantly over the first 4 days without food (Fletcher, 1984), as did the levels in rainbow trout over 5 days (Bilinski and Gardner, 1968).

Routine Aquarium Disturbance

The experiment of covering/uncovering the fish tanks was carried out to determine the effect of aquarium stress responses in plaice. The only parameter measured which altered significantly was the level of serum cortisol. There were no differences in serum glucose, NEFA or cholesterol levels when the tanks were covered and fish were undisturbed, confirming the sensitivity of plaice serum cortisol to environmental change. However, if cortisol levels are to be used in the future as an indication of a stress response, care must be taken to ensure the least possible disturbance of the fish.

Stress Responses Incurred by Agitation and Hypoxia

In channel catfish (Strange, 1980) and F. heteroclitus (Leach and Taylor, 1980), hyperglycemia occurred at a slower rate than hypercortisolism. A similar response occurs in the plaice. A significant increase in serum cortisol occurs within an hour when plaice are subjected to a mild stressor, but there is only a slight increase in the serum glucose levels. However, a significant increase

in plaice serum glucose levels occurs under more stressful conditions, although at a slower rate than that of cortisol. There is no obvious explanation for the significant hypoglycemia which occurred 3 h after the onset of exposure to the stressor in plaice from Procedure A (Experiment 5b), unless this particular group had not fully recovered from the starvation period prior to and during spawning and was suffering from low liver glycogen levels and exhaustion through lack of food. However, a similar decrease in plaice serum glucose was found in fish after reduced oxygen treatment, indicating that glucose was being utilised faster than it was being produced. If, as is possible, the liver glycogen levels were relatively low initially, this in itself would limit the amount of carbohydrate produced.

The increase in plaice plasma lactate levels in Experiment 6 after hypoxia were similar to those found in the flounder (Jørgensen and Mustafa, 1980). As in the flounder, the increase in lactate was not due to excess swimming activity, but probably to an increase in ventilation during hypoxia, causing acute or chronic stress responses in the fish. Wardle (1978) found that with the plaice during vigorous exercise, muscle glycogen levels decreased to the extent of the increase in lactic acid levels, although the amount of lactic acid released into the blood, reaching a maximum 2-3 h after exercise, was low compared to that in the muscle. It is well recognised that the mammalian heart utilises blood lactate as a source of energy, although under aerobic working conditions, NEFA are the preferred metabolic fuel. Little is known of the aerobic energy source of the fish heart, but it would be interesting to know whether the metabolism is similar

to that of the mammalian tissue as during the recovery period from strenuous activity, blood lactate in the trout may increase 5-fold within one minute (Driedzic and Kiceniuk, 1976).

There was no change in plasma ascorbic acid concentrations in hypoxic plaice. Ascorbic acid is an important dietary requirement for optimum growth in fish, but few studies have been carried out on the effects of stressors on the ascorbic acid levels in teleosts. Wedemeyer (1969b) found depletion of interrenal ascorbic acid in rainbow trout and coho salmon after mild exertion. There was no corresponding increase in plasma ascorbic acid levels, and as serum cortisol levels in the coho salmon increased, he suggested the possibility of ascorbate involvement in steroid biosynthesis.

Plaice exposed to hypoxic conditions did suffer from a mild hypercholesterolemia, showing perhaps the onset of a stress response. Plaice from Procedure B (Experiment 5b) however, showed significant hypocholesterolemia 7 h after the onset of disturbance, possibly from impaired lipid imbalance (Wedemeyer et al., 1984).

Hypercholesterolemia is present in many teleosts and is usually not associated with disease, whereas in mammals it is known to be a prime factor in the development of atherosclerosis (Farrell and Munt, 1983). The apparent rarity of atherosclerosis in fish (Farrell and Munt 1983) could be explained by the different lipoprotein with which cholesterol is carried in the blood and in the large amounts of phospholipid associated with it (Morris, 1959). A mild hypercholesterolemia has

been observed in coho salmon after handling, but not in steelhead trout (Wedemeyer, 1972).

Stress Response to Injection

Plaice serum cortisol and glucose levels had significantly increased within 24 h of an injection of adrenalin. Many species of fish, when subjected to stressors, incur an increase in plasma catecholamine levels in an initial response to the stressor (Mazeaud et al., 1977). Injection of adrenalin, as well as physical stressors, is also a well-established method of producing elevated levels of serum glucose and cortisol in teleosts, but the effect on circulating NEFA and cholesterol levels is less clear.

Plaice serum cholesterol levels were slightly elevated 24 h post-adrenalin injection, but they had declined to levels similar to control fish by 4 days and none of the other differences in cholesterol levels were significant. Hypercholesterolemia, after the administration of adrenalin however, has been reported for rainbow trout (reviewed by Hille, 1982) and the eel (Larsson, 1973).

The overall response of plaice to stressors or administered adrenalin (24 h post-injection) in these experiments has always been one of increased NEFA release. Mazeaud and Mazeaud (1981) reported that adrenalin could produce an increase or decrease in carp (Cyprinus carpio) plasma NEFA, depending on the experiment, with similar variations in NEFA levels for the other teleosts they examined.

However, injected adrenalin did produce a decrease in serum NEFA levels 4 days post-injection.

Injected endotoxin produced a decrease in plaice serum NEFA within 24 h and a significant decrease by 4 days. One possible explanation may be that lipolysis in liver tissue and the release of lipolytic endproducts are decreased following endotoxin injection. Kawakami et al. (1986) reported an increase in both plasma NEFA and cholesterol levels for the rat 12 h after endotoxin injection, but increased and decreased levels have been reported in endotoxin-treated mammals (Spitzer and Spitzer, 1983; Knowles et al., 1986).

Cholesterol levels in plaice serum did not appear to be affected by the injection of endotoxin. The i.p. injection of endotoxin, however, does produce responses in the plaice similar to that in mammals, namely hypoglycemia (McCallum and Stith, 1982) and hypercortisolism (Kimball et al., 1968). Hypoglycemia may develop due to increased peripheral glucose utilisation thus disturbing glucose homeostasis and the increase in cortisol probably to pituitary release of ACTH. Hypercortisolism has also been reported in the trout after the administration of endotoxin (Wedemeyer, 1969a). Despite these similarities, endotoxin does not have the toxic effect on plaice it has on mammals.

Relationship between Serum NEFA and Glucose

Farkas (1967; 1969), Minick and Chavin (1973) and Fletcher (1984) have all reported an inverse relationship between plasma/serum NEFA and glucose levels in teleosts. Plaice serum NEFA and glucose levels also showed a significant inverse correlation over 15 days post-capture. However, no reciprocal relationship could be found in the fish from this study after physical disturbance, adrenalin, cortisol, endotoxin or glucose injection, but a significant inverse correlation was found after hypoxia (Experiment 6; $r = -0.7906$, $N = 15$, $P < 0.001$). It is possible that the severity of the stressor affects the magnitude of the variation in metabolite levels, but the fact that these fish were not feeding, must be taken into account.

GENERAL CONCLUSIONS

Leatherland (1985) has suggested that, in some cases, changes in hematocrit, plasma electrolytes and glucose might not occur in response to a stressor but as "a specific response to a specific induced physiological state". Apart from the initial hypoglycemia in response to physical disturbance, endotoxin injection and the reduced oxygen treatment in Experiment 6, the increases in the concentration of plaice serum components could be reproduced by adrenalin injection, suggesting perhaps that, for the plaice, serum glucose, cortisol and NEFA could be used as indicators of a stress response. Although no similar increases in plaice serum components were found with injected cortisol, this is in agreement with Leatherland (1985) who suggested that secondary stress responses are not necessarily cortisol-dependent. Acute increases such as those found here are probably mediated by catecholamines (Nakano and Tomlinson, 1967; Mazeaud et al., 1977; Mazeaud and Mazeaud, 1981; Barton et al., 1986).

This investigation has indicated that plaice are placid creatures and, that only cortisol levels are affected by the disturbance of the aquarium routine. Plaice can also survive in the aquarium for some time without feeding. Only serum NEFA levels increased over the first 7 days in the aquarium, confirming its importance as an energy source for teleosts. Serum lipid concentrations in the plaice appear to be dependent on the qualitative composition of the food supply, with the lipid composition varying accordingly. Of the 8 serum components measured in the plaice, glucose, total and free cholesterol, total

lipid, phospholipid and triglyceride concentrations stabilised after 48 h in the aquarium. Cortisol and NEFA concentrations required longer and did not stabilise until day 9. This extra period of time will have to be taken into consideration for future work.

Phospholipids account for more than 50% of the lipid in plaice gonads. Although the major polar lipids in plaice gonads were similar to those reported for other marine teleosts, the major neutral lipid classes appear to depend on the sex and species studied.

In marine teleosts, the responses to stressors differ widely and no definite pattern was obvious from the results of this study on the plaice. The variations in serum concentrations of the components studied, seem to depend very much on the duration and severity of the stressor, but can mostly be reproduced by injection of adrenalin. This does however, make it very difficult to determine the criteria which can describe a "stressed" fish. Serum cortisol levels in the plaice are too sensitive to environmental change, making it an unreliable parameter to measure, and serum glucose and NEFA concentrations may increase or decrease, depending on the stressor. Not enough work was completed on the effect of stressors on plasma lactate levels, but changes in lactate concentrations might have been a more satisfactory measurement of a stress response in the plaice.

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